The function of TAR1 and the evolution of the retrograde response

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Abstract

TAR1 is a protein coding gene situated antisense to the 25S rRNA in *S. cerevisiae*. Tar1p is localized to the mitochondrial inner membrane, and expression is enhanced under conditions of respiratory dysfunction. One common cause of respiratory dysfunction in *S. cerevisiae* are selfish mitochondrial mutants known as ρ- mitotypes. ρ- mitotypes exhibit drive within the cell following sexual reproduction ; outcompeting host cells and inducing respiratory dysfunction. Respiratory dysfunction activates the Retrograde Response, which involves the expression of genes to compensate for loss of anabolic activity that accompanies respiratory dysfunction. The Retrograde Response also leads to the formation of lifespan shortening Extrachromosomal rDNA circles. Amplification of rDNA circles has the effect of increasing TAR1 at the same time as lifting transcription repression. This observation led to the hypothesis that the formation of rDNA circles was a positive effect of the Retrograde Response, and that *TAR1* may serve to ameoleriate the spread of respiratory incompetent mitochondria following sexual reproduction. In this thesis, experiments are conducted that show that *TAR1* does suppress the drive of selfish mitochondrial mutants. Additional bioinformatic analyses show that the Retrograde Response may be a recent adaption to selfish mitochondrial mutants.

CHAPTER ONE

Introduction

1.1 Selfish genetic elements exhibit drive

Selfish genetic elements are sequences of DNA that do not enhance the fitness of the host organism, but are still replicate themselves. In the model yeast *Saccharomyces cerevisiae* one type of selfish genetic element are suppressive and hypersuppressive mitochondrial DNA. In *S. cerevisiae* mitochondria are inherited from both parents during sexual reproduction (Azpiroz & Butow 1993). An effect of biparental mitochondrial inheritance is that **mito**chondrial haplo**types**- or mitotypes may be inherited from each parent at a different rate. Selfish mitotypes exhibit drive yet no longer carry functional copies of the mtDNA genes (Mehrotra & Mahler 1968). These selfish mitotypes are known as 'suppressive' mitotypes. Mating between haploid cells both carrying functional ('wild-type') mitotypes will result in an average contribution of mtDNA from each 50% over a sufficient number of crosses (Azpiroz & Butow 1993). When haploid cells carrying selfish mitotypes, and haploid cells carrying functional mitotypes are crossed, predominantly respiration incompetent mitotypes will be observed in the result diploid.

Table 1.1

Key Terms

The petite phenotype was discovered in the late 1940s (Ephrussi, et al., 1949), and shortly thereafter neutral (ρ^0) and suppressive (ρ^-) petites were identified. Much of the terminology surrounding mitochondrial inheritance in *S. cerevisiae* thus dates back to these early discoveries. Since the advent of modern molecular genetics, some of these terms have become outdated. For the sake of clarity and consistency, key terms used in this thesis are outlined below.

SGE	Selfish Genetic Element; a unit of DNA which undergoes biased transmission during sexual reproduction without providing a positive effect on organismal fitness
Drive (*d*)	Transmission during sexual reproduction at a rate greater than 50%
Mitotype	A mitochondrial haplotype; comprising a single mitochondrial chromosome (the mtDNA)
Suppressive	A mitotype that is inherited at a rate greater than $d>0.5$ during sexual reproduction
Hypersuppressive	A mitotype that is inherited at a rate close to $d>0.95$ during sexual reproduction
Petite	A yeast cell or colony of cells that are respiratory incompetent because they lack the electron transport chain. Cells carrying only ρ^0 or ρ^- mitotypes produce the petite phenotype.
ρ^-	Respiration incompetent, selfish mitotype, which exhibits drive during sexual reproduction
ρ^0	Respiration incompetent "neutral" mitotype that is non-heritable due to absence of mtDNA (for simplicity ρ^0 is referred to as a mitotype despite lacking DNA)
ρ^+	Respiration competent mitotype, sometimes referred to as 'wild type'
Heteroplasmic	Cell carrying more than one mitotype
Homoplasmic	Cell carrying only one mitotype

Suppressive and hypersuppressive mtDNA negatively affects the fitness of the whole organism under certain conditions. The mutation that results in biased transmission of these mitotypes is also correlated with loss of the genes that are encoded on the mitochondrial chromosome (Parikh, et al., 1987). These genes are essential for respiration, and thus yeast cells carrying hypersuppressive mitotypes are respiration incompetent, and can only utilize fermentable carbon sources. Yeast cells which have lost the genes of the mitochondrial chromosome form the 'petite' phenotype; characterized not only by an inability to grow on non-fermentable carbon sources but also smaller colony size and reduced growth rate compared to non-petites (Chen & Clark-Walker, 1999).

If a trait that carries a fitness cost exhibits biased transmission during sexual reproduction, a drive modifier may eventually evolve. Indeed, evolutionary countermeasures to selfish genetic elements are observed in a host of other species and it is hypothesized that responses to selfish genetic elements have played a significant role in the evolution of the eukaryote genome (Hurst & Werren, 2001) So far in *S. cerevisiae* no specific mechanism that counteracts the drive of selfish mitotypes has been detected. However, a candidate gene has been proposed to fulfil this role. In 2012 Poole and colleagues hypothesized that the rDNA gene *TAR1* may be one such response to the affliction of selfish mitotypes (Poole, et al., 2012).

1.2 The Tar1 Gene

TAR1 (**T**ranscript **A**ntisense to **R**ibosomal DNA #1) is a gene that is encoded anti-sense to the 25S rRNA gene in *Saccharomyces cerevisiae*. *TAR1* is only the second gene to be discovered overprinted on a noncoding RNA; the other being the mouse gene Ribin (Kermekchiev & Ivanova, 2001). *TAR1*

was discovered in 2002 in a screen of previously unidentified open reading frames in *S. cerevisiae*. (Coelho, et al., 2002). Expression of a putative *TAR1* orthologue has also been observed in *Kluyveromyces lactis* (Galopier, et al., 2011).

Several lines of evidence suggest that the *TAR1* is a protein-coding gene, and not one of the vast number of false positive open reading frames found antisense to non-coding RNAs. Notably, chromatin immunoprecipitation (ChIP) has identified the *TAR1* locus as a site of Pol II occupancy (Steinmetz, et al., 2006). Strand-specific dot blots confirmed that the *TAR1* ORF is transcribed, and immunoblot analysis has confirmed the existence of a protein product (Coelho, et al., 2002) (Coelho, et al., 2002). Antibody detection verified that the *TAR1* open reading frame is translated into a corresponding protein product (Galopier, et al., 2011).

Subcellular fractionation and indirect immunofluorescence show that Tar1p is localized to the mitochondria (Coelho, et al., 2002). Further work by Galopier et al. in 2011, specified the mitochondrial inner membrane as the localization site. Transmembrane localization may be directed by a predicted internal amphipathic α-helix (Galopier, et al., 2011). Two-hybrid interaction has been shown between Tar1p and Coq5p; an enzyme involved in the biosynthesis of Co-enzyme Q (Bonawitz, et al., 2008). While it was initially reported that Tar1 expression was enhanced under respiratory conditions (Bonawitz, et al., 2008), further investigation determined that while *TAR1* was expressed during respiration, synthesis of Tar1p was highest during fermentation (Galopier, et al., 2011).

TAR1 is encoded antisense to the 25S rRNA. In *S. cerevisiae* the ribosomal RNAs are encoded in a series of approximately 150 to 200 tandem repeats, each 9.1kb in size, located on chromosome XII.

Each repeat encodes the Pol I-transcribed 35S rRNA and the Pol III-transcribed 5S rRNA separated by two intergenic spacer regions; IGS1 and IGS2, that contain an autonomously replicating sequence and replication fork barrier, respectively (Linskens, et al., 1988). The rDNA array may expand and contract by changing the copy number of repeats via duplication and deletion of individual repeats. Intriguingly, the rDNA is affected by the condition of the mitochondria. Loss of respiratory function, as occurs in ρ^0 and ρ^- mitotypes, is correlated with the formation of self-replicating Extrachromosomal rDNA circles or ERCs, each carrying a single unit of rDNA. Furthermore, respiratory incompetence results in the lifting to Pol II silencing at the rDNA locus.

The mitochondrial localization of Tar1p and observed patterns of expression have led to the hypothesis that *TAR1* may play a role in ameliorating respiratory dysfunction (Bonawitz, et al., 2008). An alternate hypothesis has been put forward by Poole and colleagues (Poole, et al., 2012), that *TAR1* reduces the drive of respiratory incompetent hypersuppressive mitotypes during sexual reproduction. According to this hypothesis, expression of *TAR1* would be regulated by the mitochondrial-to-nuclear stress response pathway known as the Retrograde Response. The aim of this thesis is to test the hypothesis that *TAR1* affects the drive of hypersuppressive mitochondria, and to determine how the Retrograde Response evolved.

1.3 Mitochondrial Dynamics in *S. cerevisiae*

All eukaryotes either possess mitochondria or have secondarily lost mitochondria at some stage in their evolution (Poole & Penny, 2007). Mitochondria are comprised of two membranes enclosing a central matrix. The mitochondrial matrix and inner mitochondrial membrane is the site of the TCA

cycle and the electron transport chain. These two pathways form a nexus through which an array of biosynthetic processes branch from, including the amino acid, lipid, heme and sterol biosynthesis pathways.

Mitochondria most likely developed from the engulfment of an alpha-proteobacterium prior to the last common ancestor of the Eukaryotes: this model is known as the endosymbiont hypothesis (Poole & Penny, 2007). Over time, selection has favoured the minimization of the mitochondrial genome, and in most eukaryotes the mtDNA retains only core functions for expression and regulation of genes involved in respiration (Allen, et al., 2003).

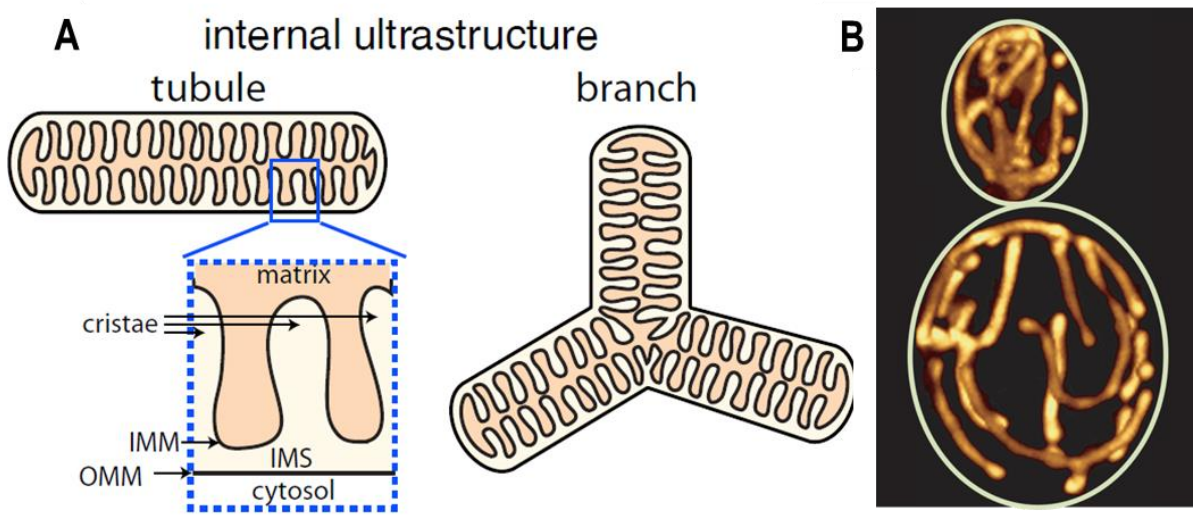


Figure 1.2: Mitochondrial network structure in Saccharomyces cerevisiae | A. *Mitochondria exist as interconnected networks comprised of tubules and branches. These networks facilitate the flow of mitochondrial components, including lipids and proteins of the inner membrane, metabolites, enzyme complexes and the mitochondrial nucleoids.* ***B.*** *Mitochondrial network in highlighted using mitochondrial localized GFP variant in a mother and daughter cell. Figure from Rafelski, 2013.*

In *Saccharomyces cerevisiae* mitochondria form branching, interconnected tubular networks throughout the cell (*see figure 1.2*). The size of the mitochondrial network within the cell can change rapidly, depending on metabolic requirements. For example respiration is correlated with an expansion of the mitochondrial network, while nitrogen starvation results in contraction through the

breakdown of unnecessary mitochondrial capacity (Devin & Rigoulet, 2004). The mitochondrial network is subject to constant fusion and fission events that result in a constantly changing network structure. A higher rate of fission will result in a fragmented mitochondrial network, while a higher frequency of fusion events will result in a more interconnected network (Rafelski, 2013). In *S. cerevisiae* the entire mitochondrial content of a cell can form a completely joined up network within 10 minutes, facilitating the rapid mixing of mitochondrial components including membrane structures and the mtDNA (Nunnari, et al., 1997).

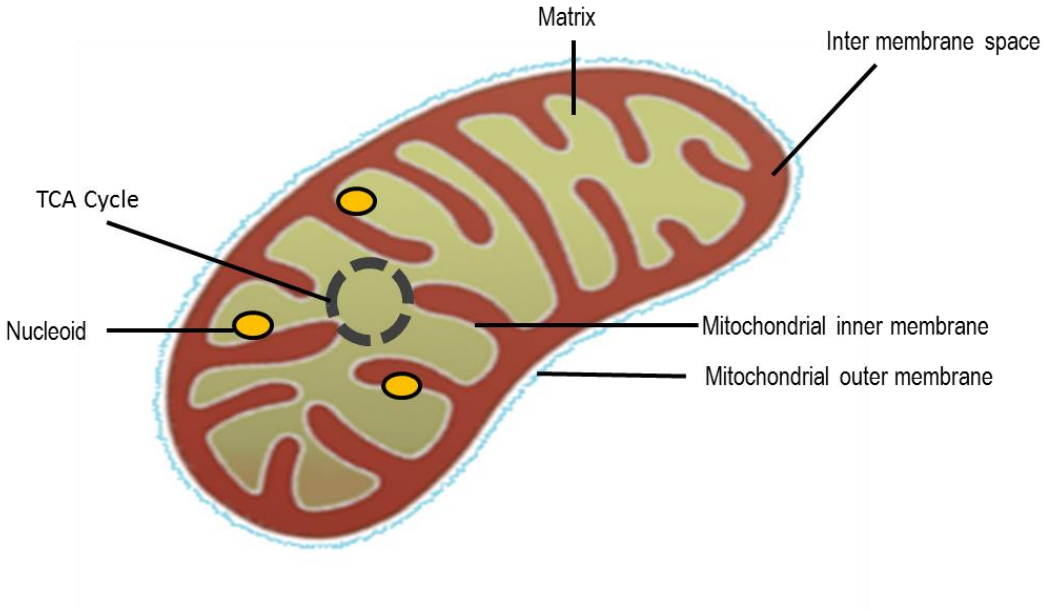


Figure 1.3: Key features of mitochondrial morphology in S. cerevisiae | *The mitochondria is composed of an outer membrane and inner membrane. The inner membrane is impermeable and cation flux is strictly regulated. Within the inner membrane is the mitochondrial matrix. The inner membrane and matrix is the site of the TCA cycle and electron transport chain.*

The mitochondrial outer membrane surrounding the organelle is comprised of roughly equal parts protein and lipid, and is punctuated by porin structures known as voltage dependent anion channels, or VDACs (Colombini, 2004).Crucially, VDACs regulate anionic metabolite flux between the cytosol

and intermembrane space, facilitating the passage of pyruvate, citrate, ATP and other anions under conditions of low membrane potential (Rostovtseva & Colombini, 1997).VDACs also regulate the flux of cations, playing a key role in calcium homeostasis within the cell. A number of ATP-dependent cytosolic enzymes are coupled to VDACs, including hexokinase which catalyses the first step of glycolysis (Shoshan-Barmatz, et al., 2010).

Unlike the outer membrane, the mitochondrial inner membrane is highly impermeable and relies on active transport of molecules, strictly regulating cation flux to provide a molecular environment conducive for oxidative phosphorylation. The space enclosed within inner membrane is called the mitochondrial matrix, a viscous environment where the reactions of the TCA cycle take place, and the mitochondrial genome, and replication and translational machineries are housed (Scheffler, 2007, pp19-30). The respiratory complex is embedded within the inner mitochondrial membrane. The large surface area of the cristae provides space for not only the electron transport chain, but also for an array of other membrane embedded mitochondrial components, including the mitochondrial genome (Mannella, 2006).

1.4 The Mitochondrial DNA

The mitochondrial genome of *S. cerevisiae* is 85.7kb (Foury, et al., 1998). It carries the genes required for F_1F_0 ATP synthase (*cox1, cox2, cox3, atp6, atp8, atp9, cytb,* and *var1*) as well as the 15S ribosomal RNA, 24 tRNAs and the RNA component of RNase P (Foury, et al., 1998). A respiratory competent *S. cerevisiae* cell will typically possess 20 to 50 copies of the mtDNA (Bullerwell, et al., 2003).

The mtDNA is packaged into discrete DNA-protein complexes known as nucleoids. The nucleoid will typically contain one or two mtDNA molecules, bounded by protein and anchored to the matrix side of the mitochondrial inner membrane (Newman, et al., 1996).The primary packaging proteins of the nucleoid are HMG (high mobility group) box proteins. Of these, *Abf2p* is most abundant, and within the nucleoid is tightly bound to the mtDNA. (Diffley & Stillman 1991). Abf2p plays a role in exposing regulatory regions of the mitochondrial genome (Diffley, 1992) and protects the mtDNA from reactive oxygen species (O'Rourke, et al., 2002). Deletion of Abf2p leads to the degradation of the mtDNA, resulting in petites carrying ρ^0 mitotypes (Zelenaya-Troitskaya, et al., 1998).

Ilv5p and *Aco1p* also play an important role within the nucleoid. *Ilv5p* is a bifunctional protein, which in addition to mediating nucleoid assembly also plays a catalytic role I n branched-chain amino acid synthesis (Zelenaya-Troitskaya, et. al., 1995). This dual function is thought to play a key role in signalling between amino acid synthesis pathways, and mitochondrial gene expression and replication as it is recruited to the nucleoid during amino acid starvation (Kucej, et al., 2008).

A third class of nucleoidal proteins are those belonging to the heat shock family of proteins. Of these, Hsp60 is the most important. Hsp60 exhibits a pleiotropic function; in addition to its structural role within the nucleoid is also responsible for folding proteins entering the mitochondrial matrix via a 14-mer torioidal chaperoinin (Voos & Röttgers, 2002). In *S. cerevisiae* Hsp60 is recruited to the nucleoid during glucose repression, and may suppress expression of mtDNA genes during fermentation (Kucej, et al., 2008).

1.5 Mitochondrial inheritance in *S. cerevisiae*

S. cerevisiae undergoes assymetric cell division via the process of budding. Because of this, mitochondria and mitochondrial nucleoids must be actively transported into daughter cells. A complex suite of proteins known as the mitochore is responsible for the movement of mitochondria from the mother cell into the bud (Boldogh, et al., 2003). The mitochore links the nucleoid to actin filaments through the inner and outer mitochondrial membranes. This ensures that not only does the daughter cell receive mitochondria, but also receives a complement of mitochondrial nucleoids (Vevea, et al., 2013).

During sexual reproduction mitochondria are inherited from both parents (Azpiroz & Butow 1993). The inheritance of two sets of mitochondria also means the inheritance of two sets of mtDNA. This presents an opportunity for genetic conflict to occur.

Shortly after cell fusion the mitochondrial networks from both parents fuse within the zygote (Nunnari, et al., 1997). Instead, the drive of ρ^- mitotypes occurs within the zygotic bud and daughter cells of the zygote, rather than during the process of sexual reproduction itself. The zygotic bud is therefore heteroplasmic. Heteroplasmy is transient however; and is only observed up to eight generations after mating (MacAlpine, et al., 2001), after which it is assumed all diploids become homoplasmic as daughter cells cease inheriting both mitotypes from the mother.

The replication advantage of selfish mitotypes arises from the heavily deleted mitochondrial genome and the amplification of 300bp sequence known as the *Ori* (Blanc & Dujon, 1980). Investigations into hypersuppressive ρ^- mitotypes have elucidated a unique mode of DNA replication, with a central role for RNA primed replication from the nonanucleotide *Ori5* sequence (MacAlpine, et al., 2001). As

even non-hypersuppressive ρ^- mitotypes are truncated, they sequester replicative machinery and produce greater numbers of shorter mtDNA molecules, inhibiting mitochondrial RNA polymerase activity on the less numerous, slower to replicate non-suppressive mtDNA molecules (MacAlpine, et al., 2001).

Suppressive and hypersuppressive ρ^- mitotypes lack the genes required for the electron transport chain, and are thus are unable to maintain the electron transport chain and respiration (Chen & Clark-Walker, 1999). Consequently yeast cells that inherit ρ^- mitotypes or ρ^0 mitotypes are restricted to utilizing ethanol fermentation and glycolysis for catabolism. Loss of the electron transport chain affects anabolic metabolism as well, through loss of function of the succinate dehydrogenase (SDH), which couples the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol (Liu & Butow 2006). Loss of SDH function blocks the completion of the TCA cycle and thus the production of downstream biosynthetic precursors. One important precursor is α-ketoglutarate. α-ketoglutarate is an essential precursor to the glutamate biosynthesis. Glutamate lies upstream of glutamine, proline, arginine and several other essential biomolecules (Crocker & Bhattacharjee, 1973). Thus the inheritance of ρ- mitotypes affects glutamate biosynthesis within the cell.

1.6 The Retrograde Response

The Retrograde Response is a shift in gene expression primarily involved in maintaining glutamate homeostasis; which is lost in cells that lack the electron transport chain (Butow & Avadhani, 2004). The Retrograde Signalling pathway is required for the Retrograde Response to be activated. The initial detector of mitochondrial dysfunction is Rtg2p, which detects a drop in mitochondrial

membrane potential (ΔΨm) that results from loss of the electron transport chain (Liao & Butow, 1993). Deletion of the nuclear encoded gene *COX4*, an essential component of the electron transport chain, also results in the activation of the Retrograde Response (Miceli, et al., 2012). Retrograde Signalling involves the translocation of the basic helix-loop-helix transcription factors of Rtg1p and Rtg3p from the cytoplasm to the nucleus (Liu & Butow, 1999) (Rothermel & Thornton, 1997). Under conditions of robust mitochondrial membrane potential Retrograde Signalling is inhibited by Mks1p phosphorylation of Rtg3p, thus precluding nuclear localization (Sekito, et al., 2002). Once localized to the nucleus Rtg1p/Rtg3p binds to a promoter element upstream of the 5' end of target genes known as an R-box (Rothermel & Thornton, 1997),(Liu & Butow, 1999). Binding to the R-box induces transcriptional activity and promotes the expression of Retrograde Response genes (Jia, et al, 1997).

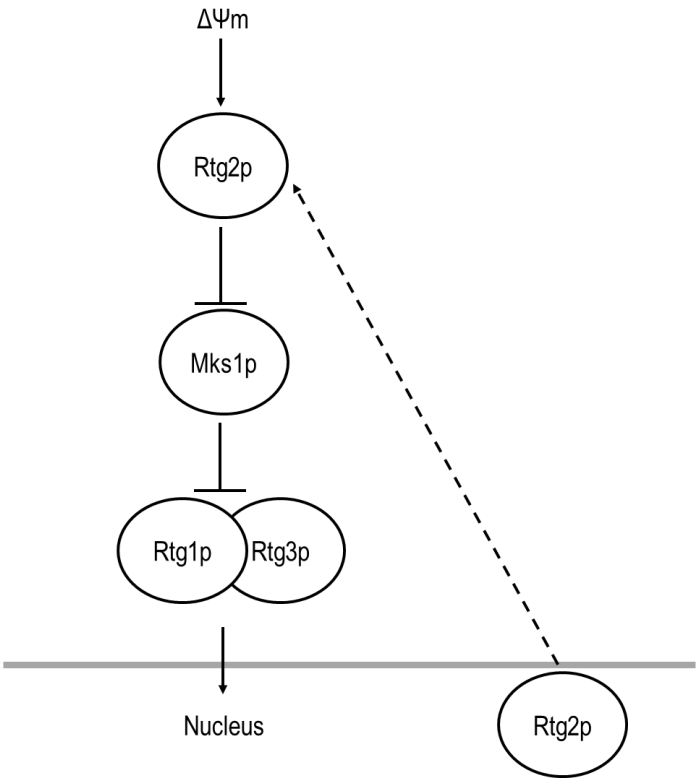


Figure 1.7: The RTG genes mediate the Retrograde Response | *Change in mitochondrial membrane potential (ΔΨm) is detected by Rtg2p, triggering the Retrograde Response. During the Retrograde Response Rtg2p moves from the nucleus to the cytoplasm, where it inhibits the phosphorylation of*

Rtg1p/3p by Mks1p. Dephosphorylated Rtg1p/3p enters the nucleus, where it promotes the expression of genes involved in mitigating the effects of mitochondrial dysfunction.

In addition to the RTG genes and Mks1p, several other proteins play a key role in regulating the Retrograde Signalling pathway. Grr1p is a positive regulator of the Retrograde Response. Grr1 is a component of the SCF complex; an ubiquitin ligase complex responsible for targeting proteins for degradation (Deshaies, 1999). Grr1p mediated ubiquitination of Mks1p allows for nuclear localization of Rtg1p/Rtg3p (Liu, et al., 2005). The 14-3-3 protein Bmh1p inhibits Grr1p mediated degradation of Mks1p, thus functioning as a negative regulator.

The Target of Rapamycin (TOR) complex is linked to the Retrograde Response through Lst8p. *S. cerevisiae* has two TOR complexes, which both mediate an array of cellular processes including controlling growth in response to nutrient availability, ribosome biogenesis and meiosis (Wullschleger, et al., 2006). Detection of glutamate by the TOR complex leads to Lst8p inhibiting Retrograde Signalling by preventing Rtg2p from interacting with Mks1p (Liu, et al., 2001) (Dilova, et al., 2002).

When the Retrograde Response is not activated, Rtg2p is localized to the nucleus, where it forms a part of the SLIK complex (Pray-Grant, et al., 2002). SLIK is a histone acetyltransferase, which regulates expression of a number of genes including those upregulated by the RTG pathway (Jazwinski, 2005). In addition to this, the SLIK complex also suppresses the formation of extrachromosomal rDNA circles (Borghouts, et al., 2004). Activation of the Retrograde Response sequesters of Rtg2p from the nucleus to the cytosol, leading to formation of ERCs (Conrad-Webb &

Butow, 1995), (Borghouts, et al., 2004). The interaction between Rtg2p and the rDNA links mitochondrial dysfunction to the *TAR1* gene.

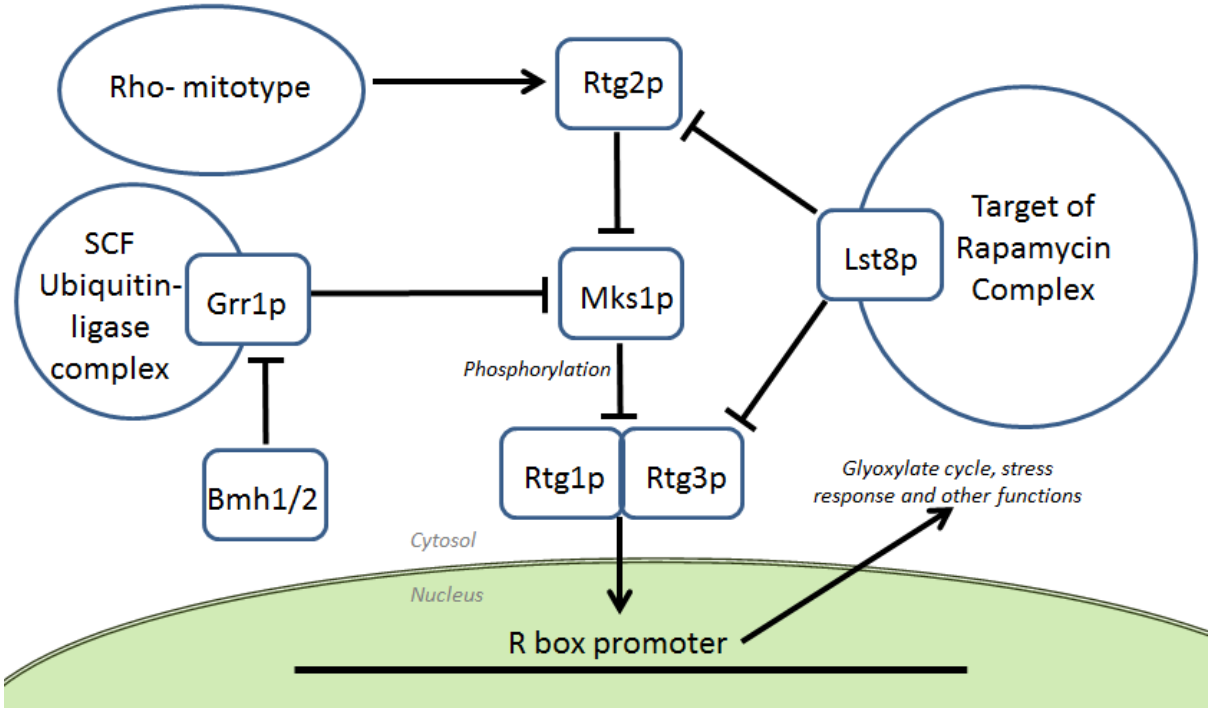


Figure 1.7: The wider Retrograde Response| *The Target of Rapamycin Complex negatively regulates the Retrograde Response via Lst8p. Grr1p tags Mks1p for ubiquitination. Bmh1p/Bmh2p blocks Grr1p from tagging Mks1p.*

1.7 The Retrograde Response enhances expression of genes involved in anaplerotic reactions

Anaplerotic reactions are biosynthesis reactions which produce intermediates of another metabolic pathway. Activation of the Retrograde Response results in the expression of genes involved in anaplerotic supplementation of the TCA cycle. One such gene is the peroxisomal isoform of citrate synthase, *CIT2*, which participates in the glyoxylate cycle (Lewin, et al., 1990).

The Glyoxylate cycle in *S. cerevisiae* is a non-mitochondrial pathway which is essential for growth on C_2 carbon sources in addition to providing an anaplerotic function in cells with a truncated TCA cycle (Lee, et al., 2011). The initial steps of the glyoxylate cycle, between citrate and isocitrate, are shared between the glyoxylate cycle and the TCA cycle. The glyoxylate cycle does not share the decarboxylation reactions of the TCA cycle, hence why it is utilized for growth on C_2 carbon sources, and thus does not produce α-ketoglutarate. Instead, citrate is exported from the peroxisome and imported into the mitochondria where it is catalyzed to α-ketoglutarate by the truncated TCA cycle.

Genome-wide transcriptional analysis of cells replete with ρ^0 mitotypes has been used to identify genes which respond to the Retrograde Response (Epstein, et al., 2001). In addition to *CIT2*, the Retrograde Response also upregulates a number of genes involved in Acetyl-CoA biosynthesis, mitochondrial and peroxisomal transporters and the genes which encode the three steps of the TCA cycle between citrate and α-ketoglutarate; *CIT1*, *ACO1*, *IDH1* and *IDH2*, as well as citrate transporters. Together these genes ensure glutamate homeostasis and thus compensate for the loss of function succinate dehydrogenase (Liu & Butow, 2006).

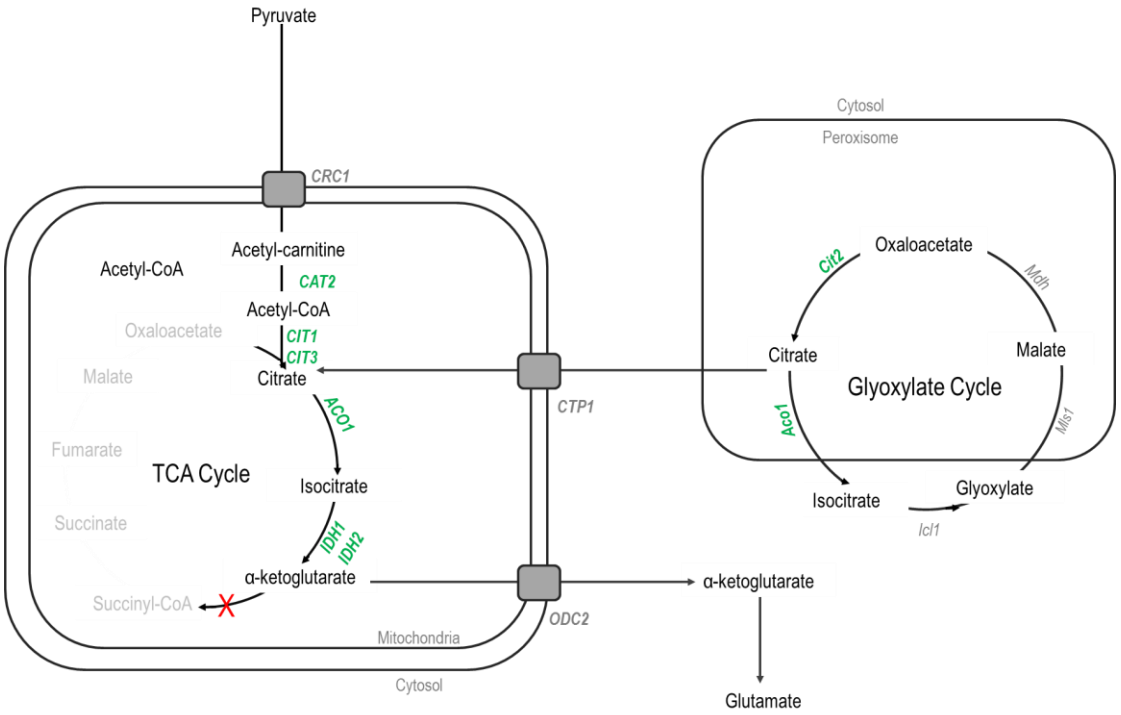


Figure 1.4: The Retrograde Response controls the expression of genes involved in anaplerotic reactions: The genes which encode the enzymes that catalyse the TCA cycle from Citrate Synthase to Isocitrate Dehydrogenase fall under Retrograde Response control in respiration incompetent cells. CIT2 is exclusively regulated by the Retrograde Response, and is localized to the peroxisomes, where it functions in the glyoxylate cycle. The glyoxylate cycle produces citrate, which is exported to the mitochondria.

1.8 The Retrograde Response prolongs replicative lifespan

In addition to its role in upregulating genes involved in glutamate homeostasis, the yeast Retrograde Response extends replicative lifespan (Miceli, et al., 2011). In *S. cerevisiae*, ageing is quantified by the number of times a cell divides. This is known as replicative lifespan (Mortimer & Johnston, 1959). Metabolic efficiency is a major determinant of lifespan and indirectly, replicative lifespan is a measure of metabolic capacity; for as the cell ages its ability to synthesize the requisite components of a daughter cell declines (Jazwinski, 2000).

While mitochondrial dysfunction has so far been discussed in the context of ρ^- and ρ^0 mitotypes, mitochondrial membrane potential also undergoes gradual decline over the course of the yeast replicative lifespan due to accumulation of damage (Eisenberg, et al., 2007). This results in the gradual ratcheting up of the Retrograde Response in aging cells (Kirchman, et al., 1999). Rtg2p is thus gradually sequestered from the nucleus into the cytosol. One consequence of this is the formation of Extrachromosomal rDNA Circles (Borghouts, et al., 2004).

1.9 Extrachromosomal rDNA Circles are associated with ageing

Extrachromosomal rDNA Circles, or ERCs arise from the rDNA array. The rDNA exists as approximately 150-200 tandemly arrayed repeats on Chromosome XII (Petes, 1979). Cellular demand for ribosomes (and thus ribosomal RNAs) varies over the course of the cells lifetime. During periods of rapid growth some 2000 ribosomes are produced per minute and the rRNA polymerase; Pol I may be responsible for up to 60% of all transcription within the cell (Warner, 1999). Within the

nucleus, the rDNA forms a part of a structure known as the nucleolus. The nucleolus is a significant nuclear structure, comprising one quarter of nucleus volume, and in *S. cerevisiae* at approximately 500 types of protein (Andersen, et al., 2005). Consistent transcription of ribosomal RNA is required for ribosomal synthesis, thus linking nucleolus integrity to virtually all aspects of cellular activity (Grigull, et al., 2004).

Each rDNA repeat is approximately 9.1kb in size, and encodes the Pol I transcribed 35S rRNA and the Pol III transcribed 5S rRNA separated by two intergenic spacer regions; IGS1 and IGS2, that contain an origin of replication (ARS) and replication fork barrier, respectively (Linskens, et al., 1988). Intra-strand recombination leads to the excision of individual arrays within the rDNA, forming ERCs. ERCs are capable of replication owing to the presence of an ARS site.

ERCs are preferentially maintained in the mother cell during budding, leading to the hypothesis that they are a senescence factor through interference with the nucleolus (Sinclair & Guarente, 1997). It was subsequently determined that ERC accumulation was correlated with reduced replicative lifespan (Falcón & Aris, 2003).

Sir2p represses of intra-strand recombination of the rDNA (Kobayashi & Ganley, 2005). This is accomplished through transcription repression of the E-pro (*see Figure X*) (Kobayashi, 2006) (Gottlieb & Easton Esposito, 1989). The E-pro is a bidirectional promoter within the spacer region of the rDNA. Transcription from E-pro disrupts the chromatin structure of the rDNA, which holds chromatids in place. Disruption facilitates intrachromatid recombination (Kobayashi & Ganley, 2005). Thus through histone silencing Sir2p suppresses the formation of ERCs (Kaeberlein, et al., 1999).

Sir2p histone silencing also suppresses all Pol II transcription from the rDNA, includes suppressing *TAR1* transcription (Fritze, et al., 1997). Thus lifting of Sir2p silencing both amplifies *TAR1* copy number as a part of the rDNA, and lifts repression of *TAR1* transcription. Activation of the Retrograde Response sequesters Rtg2p from the nucleus into the cytosol. Deletion of *RTG2* is correlated with ERC formation (Borghouts, et al., 2004), and activation of the Retrograde Response is found to correlate with ERC formation.

1.10 *TAR1* links ERCs to Mitochondrial Dysfunction

The formation of ERCs during the Retrograde Response increases *TAR1* copy number, as well as lifting suppression of expression by Pol II (Coelho, et al., 2002). *TAR1* expression is thus induced by respiration incompetent selfish mitotypes via Retrograde Signalling. The co-localization of Tar1p to the source of dysfunction has led to the proposal that *TAR1* is a nuclear encoded response to these selfish mitotypes (Poole, et al., 2012). *TAR1* as a nuclear-encoded response to selfish mitotypes would explain the apparent paradox that the lifespan elongating effects of the Retrograde Response is also induce rDNA instability and formation of ERCs.

In the following chapter a series of experiments are described which aim to test this hypothesis. In Chapter Three the evolution of the Retrograde Response is investigated. In the final chapter, the findings of these chapters are presented in the context of one another, and within the wider context of the evolution of response to selfish mitochondrial mutants.

CHAPTER TWO

Experimental Investigation into the Function of *TAR1*

2.1 Introduction

The primary aim of this thesis is to test the hypothesis that *TAR1* suppresses the drive of Rho- mitotypes during sexual reproduction. At the beginning of this chapter it is worth reiterating the salient aspects of mitochondrial dysfunction and mitotypic drive and how it relates to the experimental work described in this chapter.

A *Saccharomyces cerevisiae* cell carries between 30 and 50 individual mitochondrial genomes (Foury, et al., 1998). An individual mitochondrial genome exists as a unit of mtDNA within a nucleoid. As there are multiple units of mtDNA within the cell, multiple mtDNA haplotypes, (mitotypes) may be present within a cell. The condition of having more than one mitotype is known as heteroplasmy, while possessing only one mitotype within a cell is known as homoplasmy (Chesser, 1998). Cells may become heteroplasmic from one of two ways; either spontaneous mutations to the mtDNA may convert one mitotype to another. Or multiple mitotypes may be inherited.

In *S. cerevisiae* mitochondria are inherited from both parents during sexual reproduction. Biparental inheritance of mtDNA is rare among eukaryotes; in most species, barriers exist which ensure that only mtDNA from one parent is passed onto the zygote. A variety of mechanisms to ensure uniparental inheritance have been observed across sexually reproducing species, and for good reason. Inheritance of mitotypes from each parent leaves open the possibility for intracellular genetic conflict. Essentially, any mitotype that develops a capacity to advantage itself, or disadvantage the other mitotype, will be selected for. Uniparental inheritance precludes genetic conflict amongst the mtDNA. Biparental inheritance, conversely, encourages intracellular conflict.

Cells replete with ρ^- mitotypes produce the petite phenotype, characterized by an inability to grow on non-fermentable carbon sources, such as glycerol. The drive of ρ^- mitotypes can thus be measured

through the proportion of petite to non-petite diploid cells that arise from crosses between ρ^- petites and 'wild-type' cells. It is important to note, that hypersuppressive ρ^- mitotypes do not have a segregation advantage into the zygotic bud or into budding daughter cells. Rather, hypersuppressive ρ^- mitotypes likely have a replicative advantage, possibly due to the ability to monopolize components of the mtDNA replication apparatus (MacAlpine, et al., 2001).

The drive of ρ^- mitotypes thus occurs within the zygotic bud and the subsequent diploid daughter cells, rather than during the process of sexual reproduction itself. Heteroplasmy may be observed up to eight generations following mating (MacAlpine, et al., 2001), after which it is assumed all diploids become homoplasmic as daughter cells cease inheriting more than one mitotype from the mother. A ρ^- mitotype with a high rate of drive will reach homoplasmy earlier than a ρ^- mitotype with a lower rate of drive. The longer heteroplasmy lasts, the more likely it is that daughter cells will inherit the respiration competent ρ^+ mitotype, and not become petites.

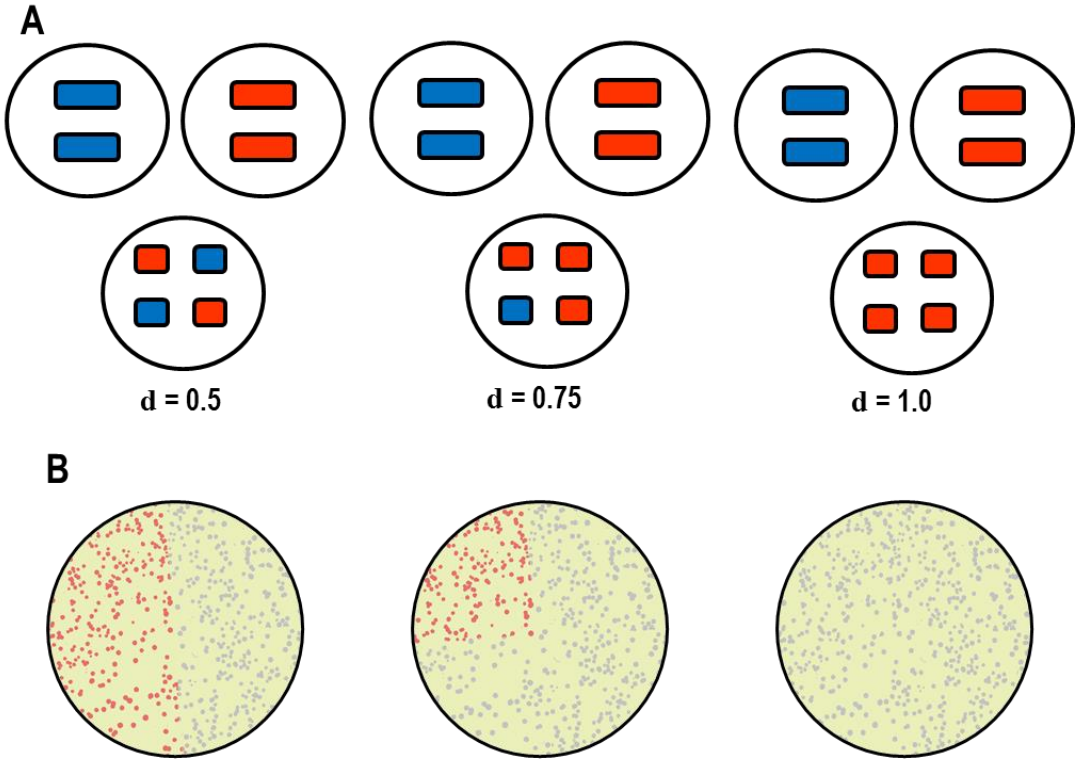


Figure 2.1: Drive of parental mitotypes will determine the phenotype of daughter cells | ***A)*** *The mitotypic composition of a diploid strain is dependent on the drive (d) of the mitotypes inherited, in this case blue = respiration competent, red = respiration incompetent. It is important to note that drive is a function of replicative capacity of the mitotype, rather than transmission advantage into the zygotic bud* ***B)*** *The mitotypic background of the zygote will determine the ratio of petites (grey) to non-petites (red) following incubation and plating onto fermentable carbon source. Over time cells will become homoplasmic each daughter cell inherits a subset of the parental mtDNA. This is a proxy measurement of drive.*

The drive of ρ^- mitotypes can thus be quantified by measuring the petite frequency which arises from a cross between a respiration competent cell, which will carry the ρ^+ mitotype, and a petite which carries the ρ^- mitotype. With this in mind, it is possible to measure factors that influence the rate of ρ^- mitotype drive during sexual reproduction in *S. cerevisiae*. This is the biological principle which the experiments described in this chapter are based upon.

TAR1 is hypothesized to be a modifier of ρ^- drive during sexual reproduction. As mentioned earlier in this section, no known mechanism in exists in *S. cerevisiae* to exclude respiration incompetent mitotypes, as is found in other eukaryotes. Selfish ρ^- mitotypes have thus developed the capacity to outcompete respiration competent mitotypes. Given the fitness cost which loss of respiratory function imparts, it is expected that a factor to mitigate the drive of ρ- mitotypes exists. *TAR1* has been hypothesized as a nuclear encoded modifier of ρ- drive. This is based upon the fact that Tar1p is localized to the mitochondrial inner membrane, and detection of mitochondrial dysfunction elicits both an increase in *TAR1* copy number and lifts repression of Pol II transcription at its locus. This hypothesis is detailed in Chapter One.

2.2 Aims

The primary aim of the experiments described in this chapter is to test if *TAR1* affects the drive of ρ^- mitotypes by using a TAR1 knockout strain *ΔTAR1*. In support of this primary aim, several additional experiments have been conducted to measure mitotype characteristics in the strains used. Firstly, background petite frequency is tested across the strains used in this work to measure the frequency of respiration incompetent phenotypes that arise in haploid strains. Secondly, the change in petite frequency over time is measured. This is important to measure, as petite frequency is used as a proxy for mitotypic drive in these experiments. Lastly, a *TAR1* overexpression plasmid was constructed, however planned experiments using this plasmid have yet to be undertaken.

2.3 Methods

2.3.1 Deletion of *TAR1*

TAR1 exists as 150 to 200 copies within the cell as a part of the ribosomal DNA array. In order to delete *TAR1* the entire rDNA array located on ChrXII has been excised, and replaced with a high copy number helper plasmid. Removal of the *TAR1* gene thus required excision of the entire rDNA array, the insertion of a premature stop codon via point mutation in the *TAR1* ORF, and then restoration the ribosomal RNA genes via a high copy number helper plasmid. This was produced and verified by Takehiko Kobayashi from the Division of Cytogenetics at the National Institute of Genetics in Japan (unpublished results). One consequence of this knockdown was a decrease in size of chromosome XII, and an atypical locus for the ribosomal RNAs.

Strains which have had the rDNA array removed, and carry the ribosomal RNA genes ectopically, are referred to as ΔΔrDNA strains.

2.3.2 Strains used

All experimental strains were kept as freezer stocks at -80^{o}C in 15% glycerol / 85% ddH_2O. Strains were awakened by defrosting and spreading onto solid rich galactose media (YPGal), and incubated for no more than 48 hours at 30^{o}C, or until colonies become differentiable by eye.

Individual non-petite colonies were selected, and placed into liquid YPGal for 24 hours approximately reaching late log phase. This correlates with an optical density at 600nm of 1.0 ± 0.2 (which correlates to 2 x 10^7 cells per ml). Each culture was diluted to 2 x 10^2 cells, and plated onto solid YPGal media, and then incubated for 48 hours.

Following incubation and differentiation of distinct petite and non-petite colonies, each plate was replicated via sterile velvet presses onto non-fermentable rich glycerol media (YPGal).

Table 2.1 Trait	Strain	Marker
ΔΔrDNA *ΔTAR1*	YAG209 α	Trp-
ΔΔrDNA *TAR*1	YAG212 a	Trp-
WT /Petite parent strain	MAT31(p) a	Leu-
WT /Petite parent strain	MAT135(p) α	Leu-
WT /Petite parent strain	Ade 101 a	Ade
WT /Petite parent strain	Ade 102 α	Ade-
ΔΔrDNA TAR1^{+}	YAG168 a	Ura-

Table 2.1 | Strains used: *Table showing strains used in these experiments. MAT31 and MAT135 were used as parents of the petite strains used in Experiment Three. Ade101 and Ade102 were used as controls for testing background petite frequency.*

2.3.3 Mating protocol

For all matings used, unless otherwise specified the following mating protocol was used.

1. 1.5ml cultures of both strain is grown overnight (12 hours) at 30^{o}C in YPGal
2. 50µl / 150µl Aliquots of each strain at a 3:1 ratio of petite : non-petite strains are added to 500ul of YPGal (this is 1:1 when mating non-petite strains with one another)
3. Cultures are incubated at 30 ^{o}C for 2 hours and rotating at 30rpm.
4. Culture is removed from incubator, and kept at room temperature (19^{o}C) overnight

2.3.4 Petite Selection

Petite strains used in these experiments were generated via spontaneous formation. Both ρ^- and ρ^0 petites are form spontaneously at the frequency cited in Experiment One.

Petites may be verified by plating on glycerol, a non-fermentable carbon source. As petites are respiration incompetent, glycerol acts as a negative selection for petites. Cells replete with ρ^- and ρ^0 mitotypes are incapable of growing on glycerol, as are nuclear petites. ρ^- and ρ^0 mitotypes can be differentiated by observing the frequency of petites in the diploid following crossing.

2.3.5 Diploid Selection

Selection for diploids was carried out using selectable markers (*see Table 2.2, strains used)* or manipulation microscopy. The use of auxotrophic markers allows for rapid and easy screening for diploids. Diploid cells may also be isolated via manipulation microscopy, which involves the selection of conjoined cells producing zygotic buds. This method allows diploids to be cultivated in rich galactose (YPGal) media, which facilitates a faster growth rate, compared to minimal media.

2.3.6 Manipulation Microscopy Selection

Manipulation microscopy was also used to select for cells undergoing mating. In *S. cerevisiae* mating cells form distinctive structures from which diploid progeny bud off, allowing for visual selection of zygotes. In order to accomplish this, 20µl mating culture was plated to YPGal media. Diploids were visually identified, and using the manipulation needle, isolated to a separate region of the plate. After 24 hours growth, colonies were picked, and grown in liquid YPGal media for 24 hours.

Diploids picked using manipulation microscopy were isolated to a separate area of the plate and grown for 12 hours. Cells from this region were then transferred to liquid media and grown to exponential phase, and plated to determine the relative frequency of petite colonies to non-petite colonies.

2.3.7 Calculating Petite Frequency

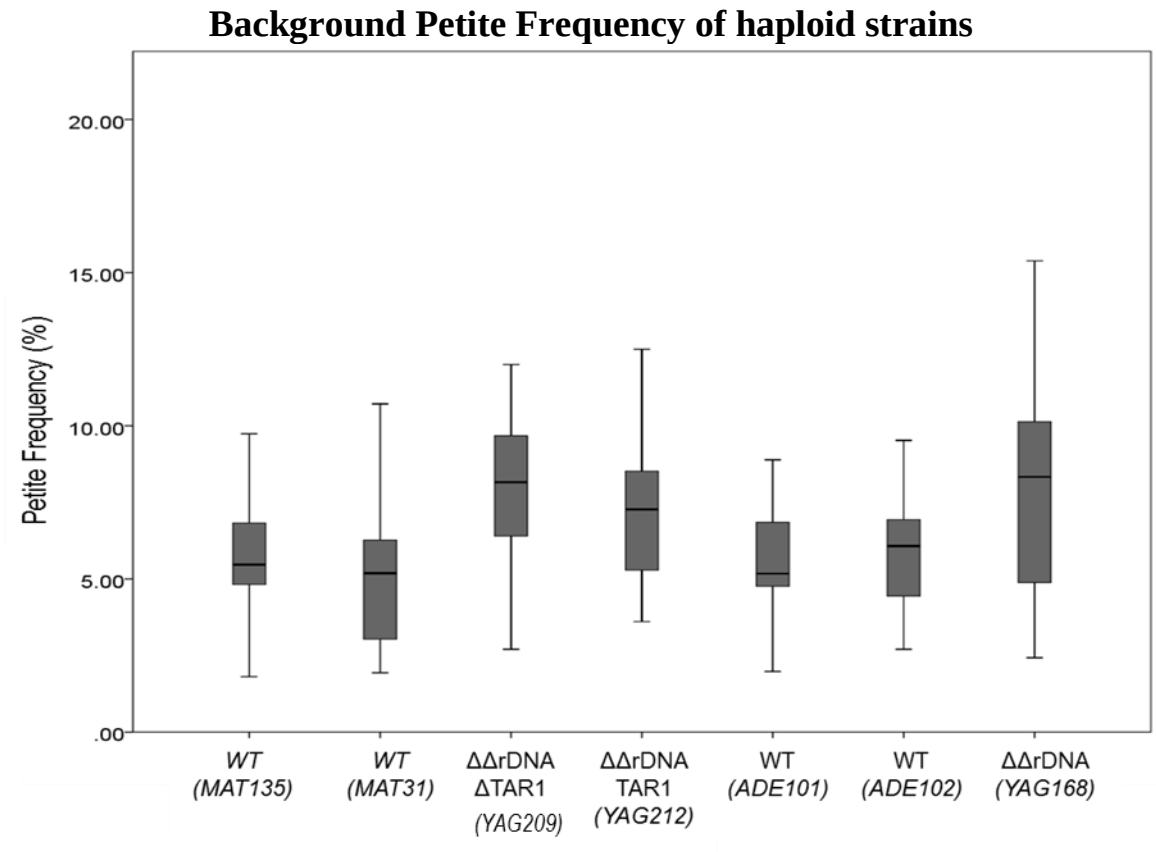
The percentage of petite colonies per plate was used to calaculate petite frequency, and a weighted average of each plate was used to calculate the petite frequency of each individual cross when multiple plates were produced per cross. Between two and five verifiable plates were produced by each cross. The average petite frequency was taken from all plates per cross. Crosses which produced petite frequencies of equal, or less than the background petite frequencies of respiration competent strains were excluded from statistical analyses; as such crosses cannot be verified as being either ρ^- or ρ^0 mitotypes.

2.3.8 Statistical Analyses

Statistical analyses were conducted using SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0).

2.4 Experiment One: Testing Background petite frequency

It was necessary to ascertain the background rate of spontaneous petite formation that occurred in strains used in these experiments. A significant difference in background petite formation in one particular strain could obfuscate phenotypic effects of *TAR1* deletion, as de novo petite formation in diploid strains may be interpreted as increased ρ- drive. Furthermore, it provided an opportunity to test if *TAR1* deletion had an effect on the *de novo* emergence of the petite phenotype. In this experiment, strains were plated onto rich galactose media, and the ratio of petite to non-petite colonies was calculated.



Graph 2.1* | *Background Petite Frequencies for haploid strains. *WT denotes the wild-type, non-petite strains of MAT135 and MAT31, and ADE101 and ADE102.*

One way ANOVAs conducted determined significant differences between ΔΔrDNA and rDNA strains (P=0.000) and individual strains (P=0.001), but not between mating types (P=0.660), *TAR1* deletion

(*P*=0.012) or strains used in crosses: MAT135 and YAG212 versus MAT31 and YAG209 (*P*=0.263). No statistically significant difference was observed between petite frequencies across ΔΔrDNA strains YAG209 (*ΔTAR1*) and YAG212 (*TAR1*)

Table 2.2 Background Petite Frequency for strains used

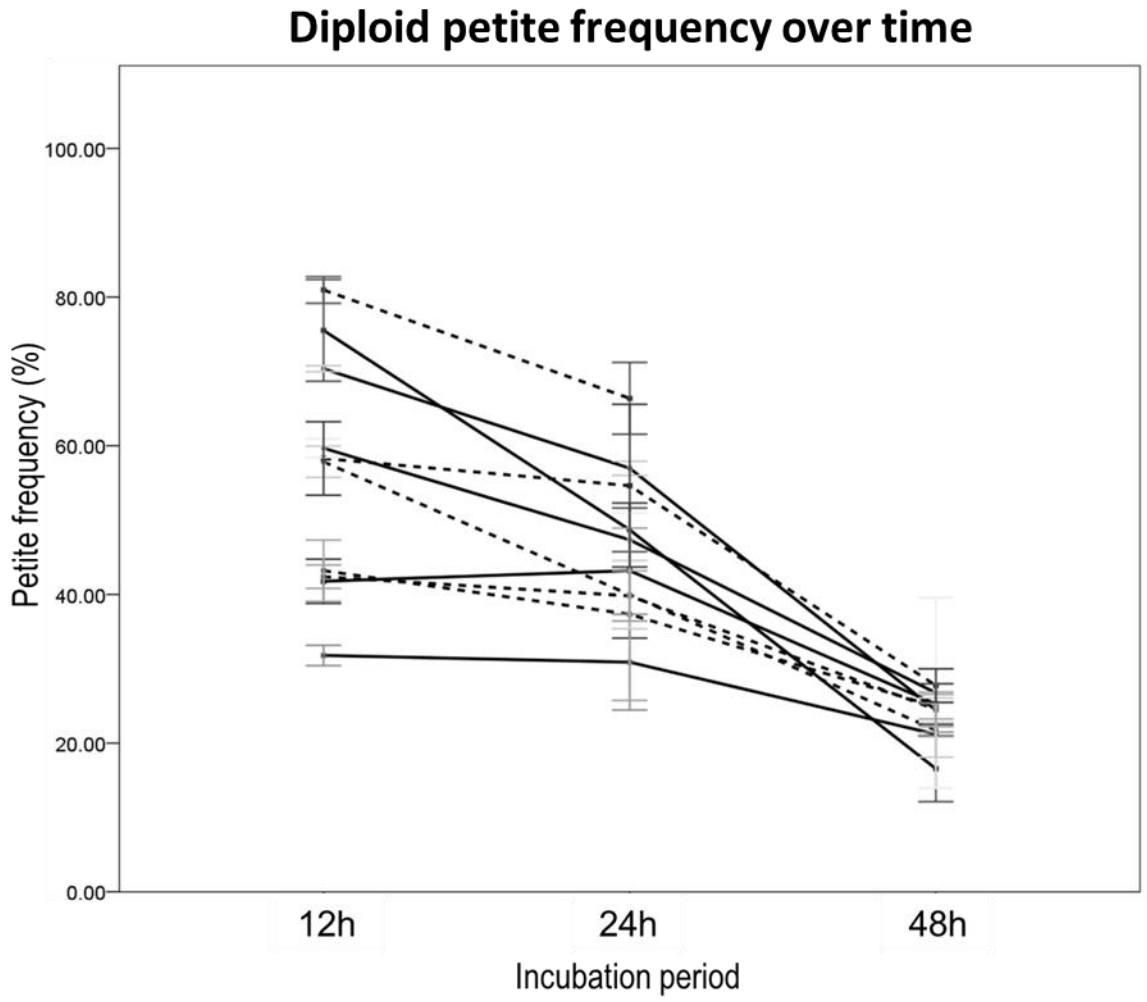
Strain	Mean	Std. Deviation	Median
WT (MAT135)	5.71	2.12	5.47
WT (MAT31)	5.15	2.27	5.19
ΔΔrDNA ΔTAR1 *(YAG209)*	8.01	2.57	8.16
ΔΔrDNA TAR1 *(YAG212)*	7.40	2.68	7.27
WT (ADE101)	5.85	2.82	5.17
WT (ADE102)	6.32	2.68	6.07
ΔΔrDNA TAR1^{+}	8.07	4.37	8.33

2.5 Experiment Two: Testing change in petite frequency over time

The earliest research into the petite phenotype recognized that these strains exhibit a reduced growth rates (Ephrussi, et al., 1949). At the same time, activation of the Retrograde Response is correlated with a prolonged replicative lifespan (Kirchman, et al., 1999). When a culture is established from a heteroplasmic parent cell, the subsequent population will be of mixed petite and non-petite cells.

In order to test if this affects the frequency of the petite phenotype over time, diploid cells were assayed for petites following incubation across different time periods in order to test how the ratio between petite and non-petite cells changed.

ΔΔrDNA TAR1- and ΔΔrDNA TAR1+ strains were mated with a ρ- petites derived from MAT31 and MAT135 respectively.



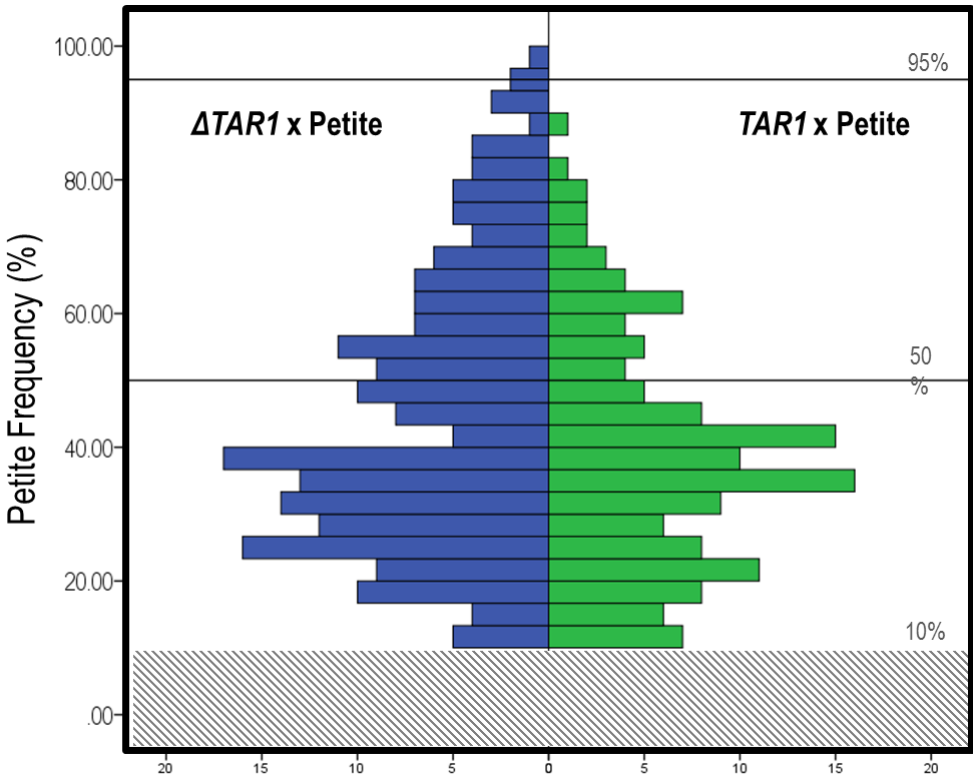
Graph 2.2 | The change in diploid petite frequency over time in YPGal media. *ΔTAR1 x petite strains (dashed line) and TAR1 x petite (solid line), (mean ± standard error) both show a decline in petite frequency which correlates with the duration of post-mating incubation.A total of ten crosses were conducted, using ρ^- petites . After 48 hours even petites resulting from strong ρ^- mitotypic drive decline to fewer than 40% of the population. Zygotes were picked with manipulation microscopy, and plated onto solid YPGal media for 12 hours, and subsequently transferred to 5ml liquid YPGal media at 30^oC for 48 hours. Samples were taken from each test after 12, 24 and 48 hours and plated onto YPGal media and incubated until differentiable petite : non-petite colonies became visible. Crosses used for this experiment were a subset of the crosses used in Experiment 3.*

The proportion of cells exhibiting the petite phenotype declines steadily from periods from 12, to 24 to 48 hours. Petite frequency is highest as cells enter stationary phase, and reaches its lowest point after 48 hours.

2.6 Experiment Three: Testing the effect of *TAR1* on mitotypic drive

In order to test the effect of *TAR1* on mitotypic drive, respiratory competent *TAR1+* and *ΔTAR1* ΔΔrDNA background strains were mated with strains carrying ρ^- mitotypes. This resulted in heteroplasmic zygotes. Mitotypic drive will occur within the zygote, and a relative measure of drive will be observable via the number of petite colonies that result from the initial mating. In essence, this experiment tests the effects that the deletion of *TAR1* from one respiraration competent (ρ^+) haploid has on the mitotypic composition of diploid progeny when mated with haploids that are respiratory incompetent (ρ^-).

Histogram of petite frequency of *ΔTAR1* x petites and *TAR1* x petites



Graph 2.3: Histogram of petite frequency between ΔTAR1 x petites and TAR1 x petites | *Deletion of TAR1 (ΔTAR1 x petites) results in a higher proportion of petites. This can be seen from the greater frequency of crosses observed with a higher percentage of petites compared to crosses between TAR1+ strain (YAG209) and petites. Crosses that resulted in fewer than 10% petite colonies per plate were excluded as they cannot be differentiated from ρ^0 petites.*

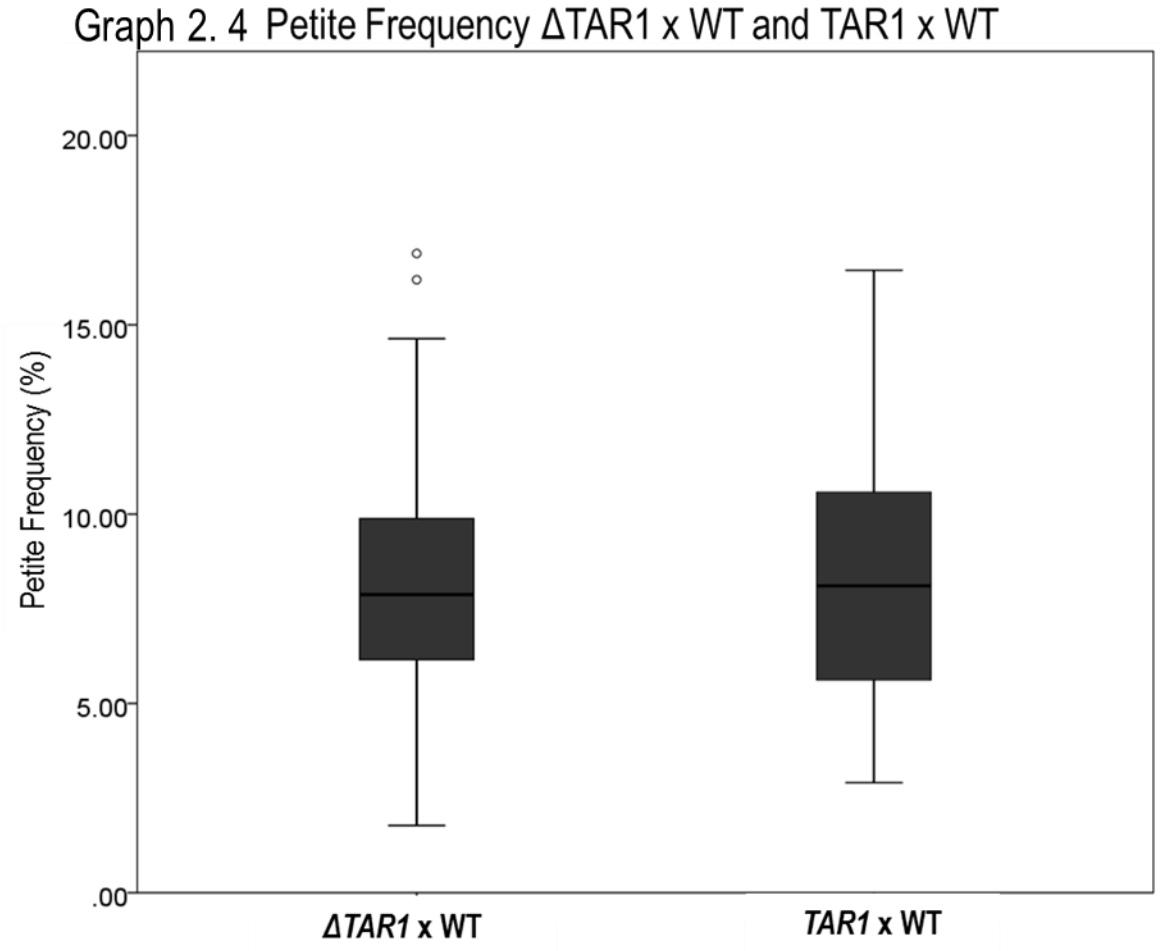
Table 2.3 Experiment Three Results		
	***ΔTAR1* x Petite strains**	**TAR1^{+} x petite strains**
Mean	44.48	38.98
Median	39.47	37.45
Std. Deviation	20.44	17.52
Variance	417.93	306.98
Range	82.9	77.27

In crosses between petite strains and the *ΔTAR1* strain, a higher frequency of diploid petites was observed that crosses between petites and the *TAR1*+ strain (Mann-Whitney U test: p-value=0.004). Crosses in which *TAR1* is deleted from the non-petite parent produce a statistically significant higher frequency of petites than strains in which *TAR1* has not been deleted.

2.7 Experiment Four: Testing the effect of *TAR1* deletion on mating between non-petite strains

The same experiments were also repeated using non-petite cells mated with petites as well. For this experiment, non-petites colonies were selected from plated freezer stocks of wild-type MAT31 and MAT135. These were incubated for 24 hours to reach late log phase, and subsequently mated with *ΔTAR1* and *TAR1* strains respectively. Unlike the previous experiment, these were plated onto media lacking Tryptophan and Leucine (-Leu , -Trp) allowing for selection using auxotrophic markers. Petite and non-petite colonies are clearly visibly differentiable after 1-3 days, and were manually counted. Replica plating onto glycerol was then used to verify respiratory incompetence. Each cross arose from a single colony arising from plates grown from freezer stocks.

Mann-Whitney U statistical test was conducted to test difference between both data sets. No statistically significant difference (p-value=0.650) was observed across between both crosses.



Graph 2.4 petite frequencies following crosses between ΔTAR1 x WT and TAR1 x WT *| Both data sets show no statistically significant difference in petite frequency (Mann-Whitney U test: p-value = 0.650).*

Table 2.3 Experiment 4: petite frequencies		
	***ΔTAR1* x WT**	**TAR1^{+} x WT**
Mean	8.17	8.4
Median	7.87	8.1
Std. Deviation	2.95	3.11
Variance	8.71	9.68
Range	15.11	13.53

2.8 Discussion

The primary aim of the experiments in this chapter was to test if *TAR1* affected mitotypic drive in *S. cerevisiae*. Results from Experiment Three found that *ΔTAR1* strains produce a greater number of diploid petite progeny when mated with petite haploids carrying ρ^- mitotypes. Deletion of *TAR1* did not affect the proportion of petite diploids following mating between two respiration competent parent strains (Experiment Four). Nor does deletion of *TAR1* affect the background petite frequency (Experiment One).

2.8.1 Limitations

It is important to highlight the limitations of the experiments presented in this chapter. Several important limittaions have had an effect on experimental design, while others are important gaps in our understanding that should be investigated before complete confidence can be placed in these results. These limitations are presented in the following section.

TAR1 expression has not been measured during the course of this investigation. Validating the expression of TAR1 is more complicated than in other genes, owing to TAR1 being antisense to the 25S rRNA (Veldman, et al., 1981). Previous research determined that Northern Blot analysis was not suitable for verifying TAR1 expression (Bonawitz, et al., 2008). However RT-PCR has been successfully used (Bonawitz, et al., 2008), (Galopier, et al., 2011).

A second important limitation was that these experiments only measured mitotypic drive indirectly by measuring the frequency of the petite phenotype following mating. This approach has its drawbacks. In Experiment Two the frequency of petites was observed to decline relative to non-petites after incubation periods of 24 and 48 hours. One reason for this may be the exhaustion of fermentable

carbon sources within the media, leading to arrested growth and eventual starvation of respiration incompetent petites. Another possible explanation for the relative decline of petites is a reduced tolerance to ethanol toxicity (Hutter & Oliver 1998). In reality the relative decline of petites may result from a combination of all of multiple factors. This observed decline is problematic as it has the potential to mask mitotypic drive, and thus future experiments should take this change in petite ratio over time into account.

Mitotypic drive may be followed through measuring the abundance of specific mitotypes at the molecular level. The drive of hypersuppressive ρ^- mitotypes has been measured using fluorescent in situ hybridization (FISH) (MacAlpine, et al., 2001). Measuring haploid and post-mating diploid frequency of mitotypes can be done by following mitotype-specific sequences. Measuring the fate of mtDNA molecules in relation to *TAR1* could be used to follow from which parent mtDNA inheritance is affected, and if *TAR* affects mitotypes that possess certain sequence motifs, such as hypersuppressive mitotypes or all respiration incompetent mitotypes.

A critical limitation which influenced the experimental design used derived from the mode of *TAR1* deletion used for these experiments. Approximately 150-200 copies of *TAR1* are present within in a wild-type cell as a part of the rDNA array (Coelho, et al., 2002). This precludes normal methods of deletion or silencing on the chromosome such as excising the entire gene or truncating the open reading frame via point mutation. Deletion of the entire rDNA array, and its replacement with a helper plasmid was used to produce the ΔΔrDNA.

As has been shown in Experiment One, ΔΔrDNA strains have a statistically significant difference in background petite formation (ANOVA P=0.000). This may be a result of the strains used in these

experiments having a higher propensity of spontaneous petite formation. Alternately, there may be a relationship between the rDNA array and a propensity towards producing the petite phenotype.

Petite *ΔΔrDNA* strains were found to have an exceptionally low mating efficiency. The handful of crosses that were carried out on plates using auxotrophic markers failed to produce colonies large enough to allow differentiation between petite and non-petite colonies by eye and did not successfully transfer to glycerol for testing of respiratory competence. Zygotic buds were produced at such a low frequency that none were observed through the microscope, thus precluding isolation of diploids onto rich carbon media via manipulation microscopy. This prevented investigation into the effect of *TAR1* deletion within the petite parent (or both parents) during sexual reproduction.

It is unclear if this phenomenon is unique to the strains used, or if there is some aspect of yeast cell biology which prevents sexual reproduction in respiratory incompetent *ΔΔrDNA* backgrounds. Given the links between the rDNA, nucleolar structure, mating type silencing and the mitochondria, it is plausible that mating is somehow inhibited for cells exhibiting both these traits. This effect was observed for both *TAR1+* and Δ*TAR1* strains.

An additional issue arising from the mode of *TAR1* deletion resulted from the different mating types of the strains carrying the Δ*TAR1* and *TAR1+* helper plasmid (strains 209 and 212). The solution to this problem was to produce a large dataset to ensure statistical robustness for a comparison between both tests and then use nonparametric statistical methods to test if there is a difference between both data sets.

2.8.2 The function of *TAR1*

These results suggest that *TAR1* has an effect on mitotypic drive during sexual reproduction, but does not affect the spontaneous formation of the petite phenotype. What does this mean from a biological perspective?

When haploid *S. cerevisiae* cells mate, the cytoplasm, including the mitochondria, mixes. Subsequently, mitochondria are actively transported into a bud that forms from the zygote. If Tar1p affects this process, then it must have an effect at this point in the mating cycle. Following this process, meiosis will occur and the rDNA array, including *TAR1*, will be incorporated into the daughter cells, eliminating any effect of *TAR1* deletion. These results therefore indicate not only an effect of *TAR1* deletion on the drive of respiratory incompetent mitotypes, but also hint towards the timing during which *TAR1* plays a role in mitochondrial drive. How could *TAR1* affect the mitotype drive during sexual reproduction according to the results presented in this chapter? Three models of how the function of *TAR1* could explain these results are presented.

1. *TAR1* excludes mtDNA from the mate during sexual

TAR1 may exclude mitotypes from the mate from entering or replicating within the zygotic bud, therefore giving its own mtDNA (ρ^+) an advantage. Deletion of *TAR1* from one mate enhances the transmission of Tar1p from the opposite mate. For *Tar1p* to exclude its partner's mitochondria, *TAR1* would have to be expressed during mating. This could be triggered by the exposure to an active Retrograde Response in the mate following cell fusion during mating. In aged cells, the Retrograde

Response would be already activated as mitochondrial membrane potential falls over the cells lifetime (Jazwinski, 2005). Age related decline in mitochondrial membrane potential would result in constitutive *TAR1* expression.

2. *TAR1* inhibits the export of damaged mtDNA

A second model that fits with these results is that *TAR1* may endogenously hinder the movement of ρ^0 and ρ^- mitotypes into the zygote, rather than affecting the transmission of its mates mtDNA. When *TAR1* is deleted from a respiration competent haploid that is subsequently mated with a petite carrying ρ^- mitotypes, the movement of a small proportion ρ^0 and ρ^- mitotypes within a heteroplasmic cell may no longer be suppressed. In such a scenario the observed increase in petite progeny results from a greater number of ρ^0 and ρ^- mitotypes being inherited from the non-petite parent, rather than the drive of ρ^- mitotypes from the petite parent.

The mechanism of mitochondrial transport into the zygotic bud is not fully understood (Vevea, et al., 2013). However assuming a mechanism similar to that which occurs during budding, then such a role for *TAR1* would be observed in Experiment One, in that deletion of *TAR1* would result in an increased background petite frequency in haploid strains. This was not observed, however.

3. TAR1 inhibits mtDNA recombination within the zygote

Tar1p is localized to the mitochondrial inner membrane (Galopier, et al., 2011). During mating fusion of the mitochondrial network follows cell fusion (Nunnari, et al., 1997). One effect of cell fusion is a mixing of mitochondrial components, including the mitochondrial nucleoid which carries the mtDNA. mtDNA molecules readily undergo recombination with one another (Shannon, et al., 1972). If Tar1p

inhibited the movement of the nucleoid within the inner mitochondrial membrane, it would reduce the rate of mixing of ρ^+ mtDNA with ρ^- mtDNA. Recombination between both mitotypes results in the conversion of ρ^+ to ρ^- mtDNA. Slowing the rate of this recombination would result in an increased number of buds being produced containing ρ^+ mitotypes until recombination homogenized the mtDNA complement of the cell resulting in respiration incompetence. This model is further discussed in Chapter Four, section 4.2.1.

2.8.3 Future Directions

Each of these models is predicated on certain assumptions made during the course of these experiments. For any future investigation into the function of *TAR1* is it crucial that these assumptions are experimental validated?

An alternate means of *TAR1* knockout

An ideal knockout method for *TAR1* would not induce major changes to the nucleolus or potentially interfere with metabolism or other essential cell processes. A post-transcriptional knockdown method using artificial snoRNAs is one potential option. SnoRNAs (Small Nucleolar RNAs) guide nucleotide modifications, including ribose methylation. Artificial C/D box snoRNAs have been developed to suppress gene expression by nucleotide methylation (Richter, 2013). Such a technique would be appropriate in suppressing TAR1 expression by methylating an early codon, and thus preventing ribosome binding.

Investigating the effect of TAR1 overexpression

The effects of *TAR1* overexpression ρ^- mitotype drive remains to be tested. Constitutive expression of *TAR1* during growth in non-petite cells may also be worthwhile investigating. As a part of this experiment an overexpression plasmid was constructued, however due to time constrains it was not used in experiments.

Overexpression plasmid was constructed on the pPS2037 vector backbone. This vector carries the promoter from the PGK1 gene, a highly expressed phosphoglycerate kinase gene. The PGK vector also possesses ampicillin resistance, allowing for selection in transformed E coli. This is based on the plasmid developed by Brodsky et al., 2000. Furthermore, PS2037 is a high copy vector, with a URA3 selective marker, imparting both positive and negative selection capacity for the plasmid

Two versions of the *TAR1* overexpression plasmids were constructed, with the only difference being one possessing a myc-tag at the 3' end (C-terminus) of the *TAR1* gene, linked by a poly-alanine tail. The presence of the myc-tag allows verification for the expression of the *TAR1* gene using antibodies. Due to the small size and likely transmembrane localization of the *TAR1* gene, there is a possibility that such an extension to the protein may impede the function of Tar1p, so a plasmid carrying the *TAR1* gene without the myc-tag was also constructed to ensure expression of a normal protein product. Under identical environmental conditions, both plasmids should express *TAR1* at a similar rate.

In future experiments this will be used to test the effect of TAR1 overexpression on background petite frequency, as well as observing ρ- mitotypic drive. As overexpression of TAR1 does not interfere with the rDNA as does deletion, the effect of TAR1 overexpression in the drive of ρ- mitotypes can be observed from both parents during crosses.

2.8.3 Conclusion

The results from this chapter support the hypothesis that *TAR1* depresses the drive of selfish mitochondrial mtDNA. Important limitations of these experiments which need to be considered, and the future directions for research are highlighted. Three possible mechanisms of TAR1 action are described. This is further expanded upon in Chapter Four, section 4.2.1.

CHAPTER THREE

The Evolution of the Retrograde Response

3.1 Introduction

Results presented in Chapter 2 support the hypothesis that *TAR1* suppresses the drive of respiration incompetent ρ^- mitotypes during sexual reproduction. A corollary of this observation is that *TAR1* is expressed as a part of the Retrograde Response. The inheritance or spontaneous formation of ρ^0 and ρ^- mitotypes leads to the activation of the Retrograde Response, as these mitotypes lack the genes required for the electron transport chain thus inducing a decline in mitochondrial membrane potential (Liao & Butow, 1993). Loss of the electron transport chain results in the truncation of the TCA cycle through the loss of Succinate dehydrogenase, which is coupled to the electron transport chain. The truncated TCA cycle leads to the depletion of oxaloacetate, and subsequently, α-ketoglutarate. α-ketoglutarate is an essential precursor to glutamate. Thus while catabolic reactions are uninhibited in cells with of ρ^0 and ρ^- mitotypes while growing on fermentable carbon sources, anabolic reactions are affected. The Retrograde Response ameliorates anabolic dysfunction in *S. cerevisiae* cells replete with dysfunctional mitochondria by upregulating genes involved in anaplerotic pathways.

The Retrograde Signalling pathway is discussed in detail in Chapter One. Several key points are worth repeating however. The Retrograde Response is dependent on three positive regulators; the Ppx/GppA phosphatase family protein Rtg2p, which is the initial detector of mitochondrial dysfunction (Liao & Butow, 1993), and the basic helix-loop-helix transcription factors Rtg1p and Rtg3p, which exist in the cytosol and nucleus as a dimeric transcription factor (Liu & Butow, 1999). Mks1p is a negative regulator; acting to inhibit the nuclear localization of Rtg1p/Rtg3p via phosphorylation (Sekito, et al., 2002). Grr1p, as a part of the SCF Ubiquitin-ligase complex, is a secondary positive regulator, promoting the degradation of Mks1p (Deshaies, 1999). Bmh1p/2p is a negative regulator of retrograde signalling, inhibiting Grr1p ubiquination of Mks1p (Liu, et al., 2005).

Lst8p is also a negative regulator of retrograde signalling. Lst8p inhibits RTG signalling at both Rtg2p detection of mitochondrial dysfunction, and the nuclear localization of Rtg1p/2p. Lst8p is a part of the Target of Rapamycin TORC2 complex (Liu, et al., 2001).

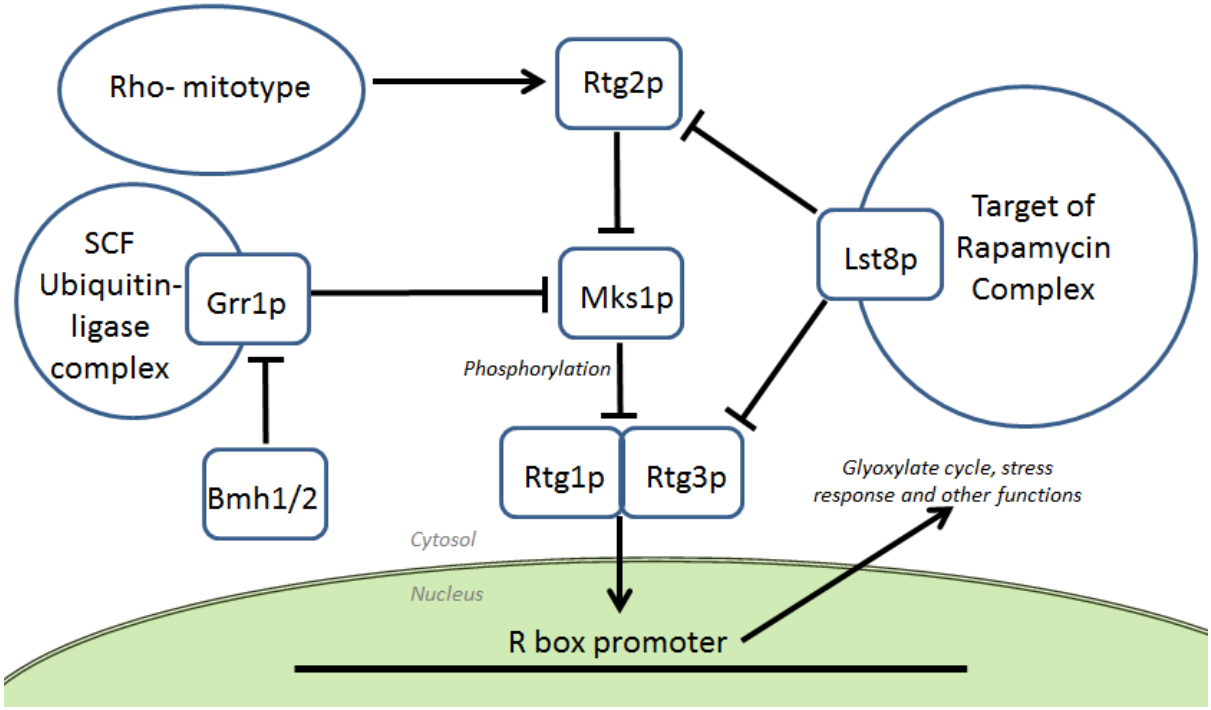


Figure 3.1: The S. cerevisiae Retrograde Signalling Pathway | *A drop in mitochondrial membrane potential ($\Delta\Psi_m$) is detected by Rtg2p, leading to Rtg2p inhibition of Mks1p phosphorylation of Rtg1p. The dephosphorylated Rtg1p/Rtg3p dimer moves into nucleus, where it binds to the R-box at the promoter site of Retrograde Response genes. Grr1p promotes degradation of Mks1p, which in turn is inhibited by Bmh1p (dimeric with Bmh2p). Lst8p as a part of the Target of Rapamycin complex (TOR) inhibits the Retrograde Signalling pathway both upstream and downstream of Mks1p.*

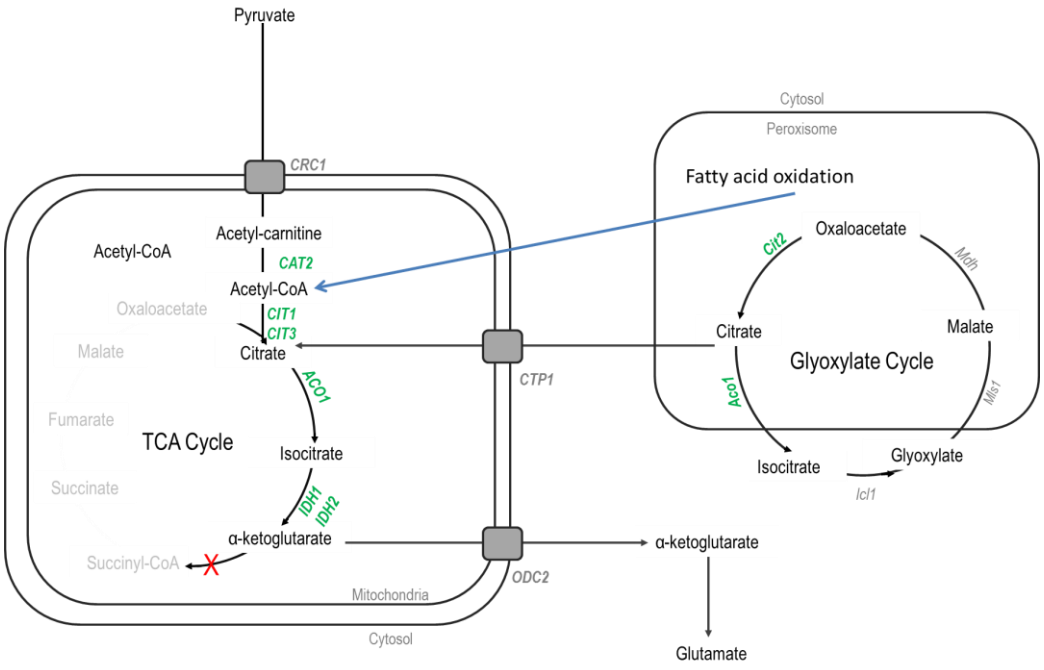


Figure 3.2 Functions of the Retrograde Response in S. cerevisiae **|** *The Retrograde Response is involved in the glyoxylate cycle (right) and the TCA cycle (left). Genes regulated by the Retrograde are in green text. TCA cycle dysfunction resulting from loss of the TCA cycle enzyme Succinate Cycle is illustrated by the red x.*

Much of the current understanding of the Retrograde Response comes from genome-wide transcriptional analysis of respiration incompetent cells, including transcriptional responses to the deletion of the RTG genes (Epstein, et al., 2001). Two types of change in expression are observed in Retrograde Response genes. One group of genes are ordinarily expressed in cells with functional mitochondria, and during the Retrograde Response transcriptional control falls under Rtg1p/Rtg3p. The second group are only expressed during activation of the Retrograde Response. Of note, CIT2 falls under this second category, and is involved anaplerotic reactions that supplement the TCA cycle.

The Retrograde Response is activated in the respiration incompetent petite phenotype. While the petite phenotype may be induced through nuclear mutation it most frequently arises through the spontaneous generation and inheritance of ρ^0 and ρ^- mitotypes (Chen & Clark-Walker, 1999). Petite

positive species; those in which the petite phenotype is observed, are found across the Saccharomycetaceae (Fekete, et al., 2007), suggesting a role for the Retrograde Response in these petite positive Saccharomycetaceae species. Outside of this grouping, the petite phenotype has been induced in *C. albicans* (Cheng, et al, 2007) as well as the more distantly related Taphrinomycotina yeast; *Schizosaccharomyces pombe* (Haffter & Fox, 1992) In both cases, the petite phenotype was only induced through nuclear mutations, rather than loss of the mtDNA, suggesting it is an unrelated phenomenon to the cytoplasmic petites of *S. cerevisiae* and kin. *RTG2* homologues from *Ashbya gossypii, Candida glabrata, Vanderwaltozyma polyspora* and *Kluyveromyces lactis* were found to functionally complement defective Rtg2p in mediating the Retrograde Response in *S. cerevisiae* (Ünlü, et al., 2013). Aside from this, very little is known about the Retrograde Response in other yeast species.

This begs the question as to whether or not the Retrograde Response is an evolutionary response to ρ^0 and ρ^- mitotypes, or a general stress response pathway which bolsters anabolic function that happens to support glutamate homeostasis in petite cells.

The aim of this chapter is to determine if the Retrograde Response has co-evolved with the petite phenotype, or if it as a more generalized stress response pathway. This will be tested using phyletic profiling and phylogenetic analysis of Retrograde Response genes.

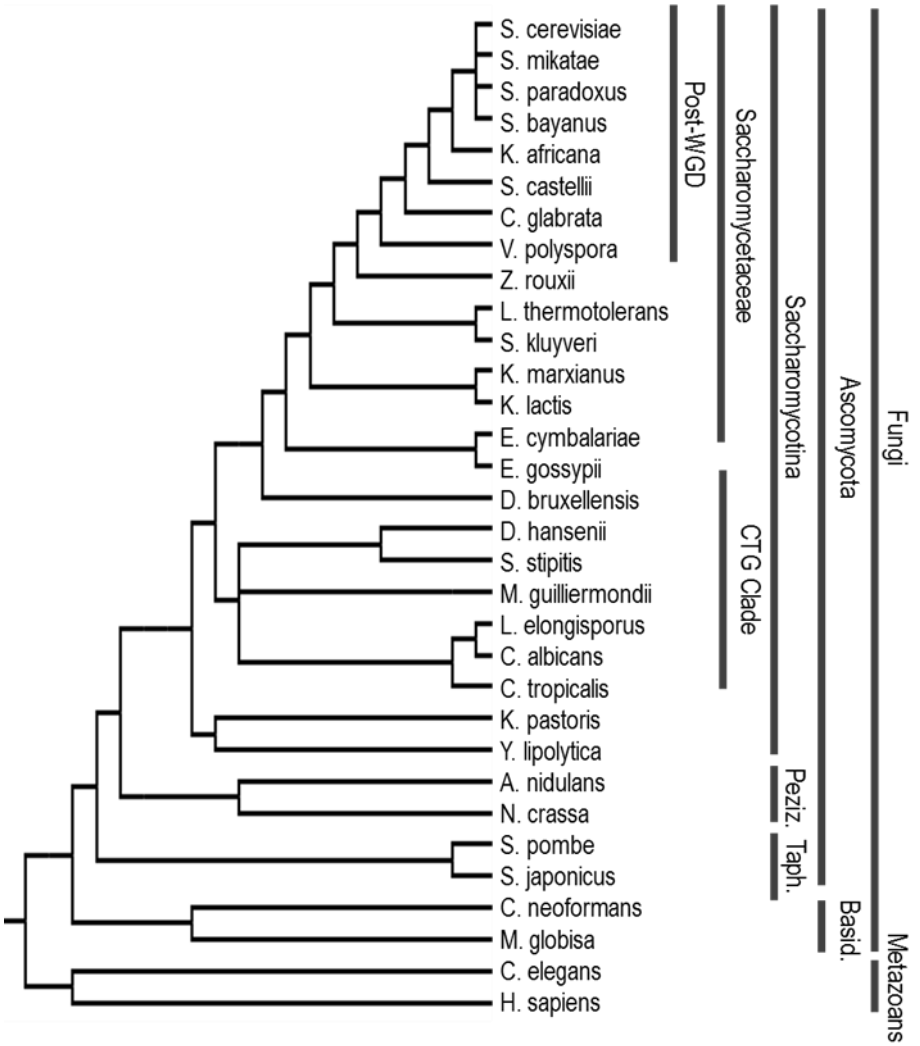


Fig3.3: Cladogram of representative Opisthokont species | *the species used for figures 3.4, 3.5, and 3.6 are shown here within their respective taxonomic divisions. Cladogram based upon phylogenies constructed by Suh, et al., 2006 and James et al., 2006. (Peziz. = Pezizomycotina, Basid. = Basidiomycota).*

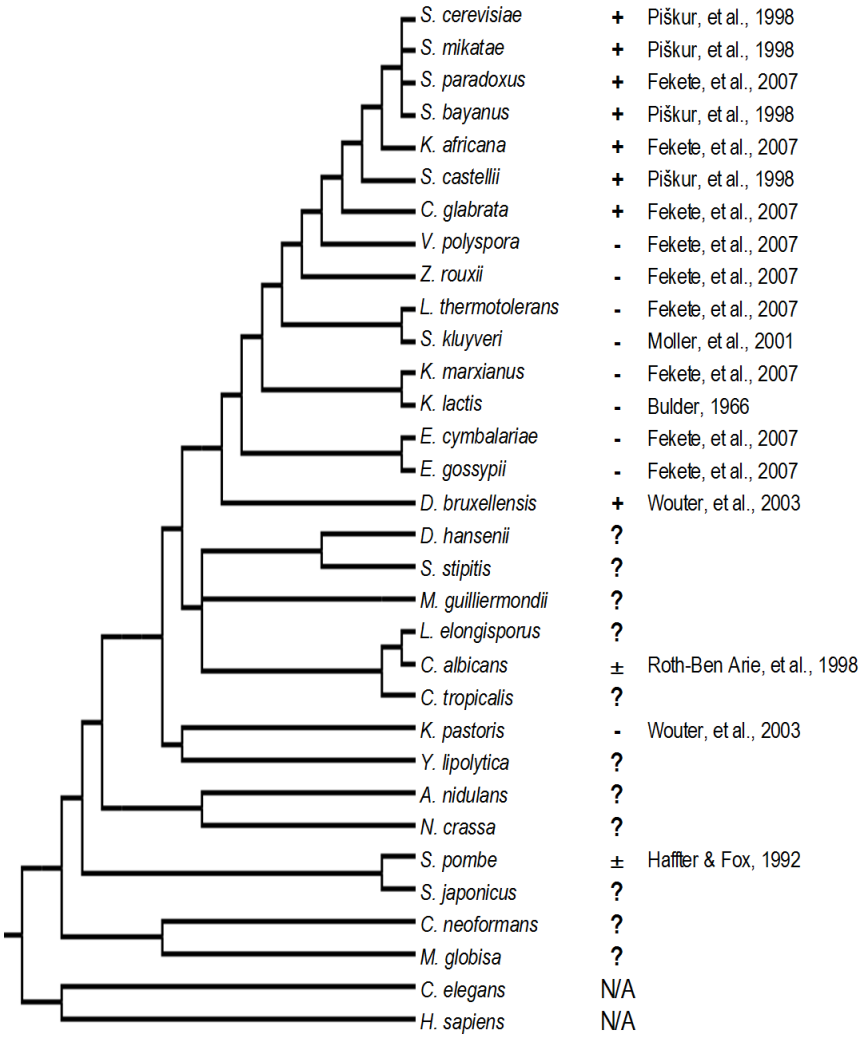


Figure 3.4: Distribution of the Petite Phenotype in selected yeasts | *+ indicates petite positive species, - petite negative species, **?** Uncertain, and ± indicates only nuclear petites in experimentally modified strains have been observed.*

3.2 Methods

3.2.1 Phyletic Profiling

Nucleotide and amino acid sequences for each gene from *Saccharomyces cerevisiae* s288c were retrieved from the Saccharomyces Genome Database.

Species selected for phyletic profiling based upon their phylogenetic distribution to ensure coverage of all main Ascomycota subphyla. A proportionally higher number of species are from the Saccharomycotina, to provide greater detail to this taxon. Only one strain per species was selected.

Amino acid sequences were searched against the UniProt database (UniProt Consortium, 2008) using Hmmer3 (Eddy, 2009). Strains were also searched using BLASTp against species specific datasets that are not included as a part of the Uniprot database. The top sequences with a high degree of sequence similarity were retrieved, provided they had an e-value greater than $1e^{-10}$.

Reciprocal BLASTp searches were carried out against the *Saccharomyces cerevisiae* s288c (NCBI taxid:559292) reference genome of the top hits for each search from each species, to indicate orthology. If the top hit from a given species for a given gene was the original *S. cerevisiae* query gene then it was considered a putative orthologue for the purposes of phyletic profiling.

3.2.2 Phylogenetic Analysis

Phylogenetic trees were constructed using PhyML (Guindon, et al., 2003) using the Phylogeny.fr server (Dereeper, et al., 2008). Gblocks (Castresana, 2000) was used for alignment curation. Maximum Likelihood trees were constructed using sequences aligned by MUSCLE (Edgar, 2004).Jones-Taylor-Thornton model was used (Jones, et al., 1992).

3.3 Phyletic Profiling of the Retrograde Response

Phyletic Profiling of Retrograde Signalling pathway genes

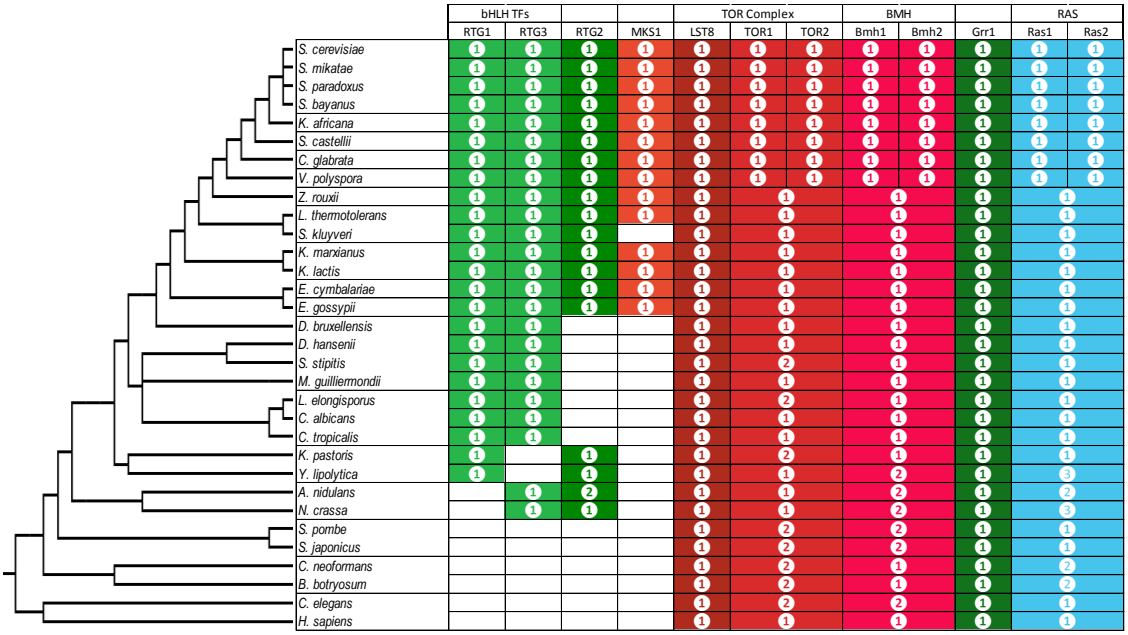


Figure 3.5: Distribution of the RR genes in the Ascomycota: *two distinct patterns of gene distribution are seen: the RTG genes and MKS1 and not ubiquitous, while the peripheral regulators, including TOR1/2, BMH1/2, RAS1/2 and GRR1 are highly conserved. Numbers show number of isoforms of the gene present in each species. Green boxes indicate positive regulators, red boxes indicate negative regulators. Orthologues were identified using reciprocal BLASTp.*

Phyletic profiling results show that for *RTG2*, it has been acquired most likely prior to the divergence of the *Saccharomycetes* yeasts from the rest of the Ascomycota. Within the Saccharomycotina *RTG2* appears to have been secondarily lost within the CTG clade.

The other two RTG genes; *RTG1* and *RTG3* show a complex pattern of phylogenetic distribution based upon this comparative analysis. Among the Saccharomycotina, RTG1 is universally conserved, while *RTG3* is absent only in *Y. lipolytica* and *D. bruxellensis.*

The negative regulator *MKS1* is present only within the Saccharomycetaceae yeasts. BLASTp searches identified no putative orthologues outside of this taxon. Only one copy of each of the RAS, TOR and BMH genes was identified in the pre-Whole genome duplication yeasts, suggesting that these genes have retained paralogs following the whole genome duplication event.

Evolution of genes upregulated by the Retrograde Response

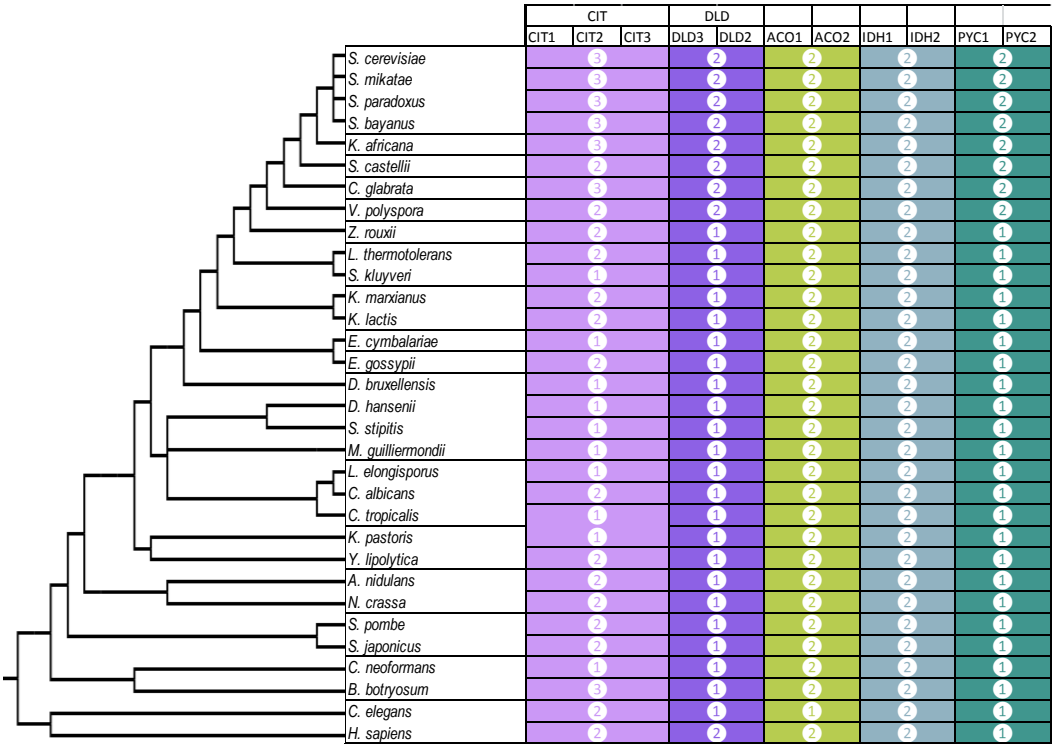


Figure 3.6: Selected Retrograde Response target gene duplicates *Reciprocal BLASTp searches were used to identify orthologues for each gene. This cursory search of Retrograde Response target genes identified that ACO1/2 and IDH1/2 have retained two copies throughout the fungi and metazoans. DLD, PYC and CIT have undergone duplication events consistent in timing with the Saccharomycetaceae whole genome duplication event.*

CIT1/2, *ACO1/2* and *IDH1/2* encode the enzymes which form a part of the TCA cycle that is regulated by the Retrograde Response in respiration incompetent cells (Epstein, et al., 2001). The section of the TCA cycle controlled by the Retrograde Response catalyzes the reactions from citrate to

α-ketoglutarate. *DLD3* and *DLD2* encode two isoforms of D-lactate dehydrogenase, of which *DLD2* shows a strong increase in expression following activation of the Retrograde Response (Chelstowska, et al., 1999). *PYC1* and *PYC2* are differentially expressed isoenzymes of pyruvate carboxylase, which converts pyruvate to oxaloacetate (Walker, et al., 1991). *PYC1* shows a strong response to activation of the Retrograde Response (Epstein et al., 2001).

3.4 Phylogenetic Analysis of Citrate Synthase

Citrate synthase is an enzyme that catalyses the condensation reaction between acetyl-CoA and oxaloacetate producing citrate. In *Saccharomyces cerevisiae* there are three citrate synthase genes: *CIT1*, *CIT2*, and *CIT3*. Cit1p operates as a part of the TCA cycle, localized within the mitochondrial inner membrane. Cit3p is likewise found in the mitochondrial inner membrane, and is capable of both catalysing the citrate synthase reaction activity, and also functions as methylcitrate synthase, which catalyses the condensation of propanoyl-CoA and oxaloacetate to produce (2R,3S)-2-hydroxybutane-1,2,3-tricarboxylate (Graybill, et al., 2006). When *CIT1* is deleted *CIT3* may compensate. *CIT2*, on the other hand, is not localized to the mitochondria. Instead, *CIT2* is peroxisomal, and is involved in the glyoxylate cycle (Lee, et al., 2000). *CIT2* expression is controlled by the Retrograde Response; activation of the Retrograde Response results in drastic upregulation in *CIT2* (Epstein et al., 2001).

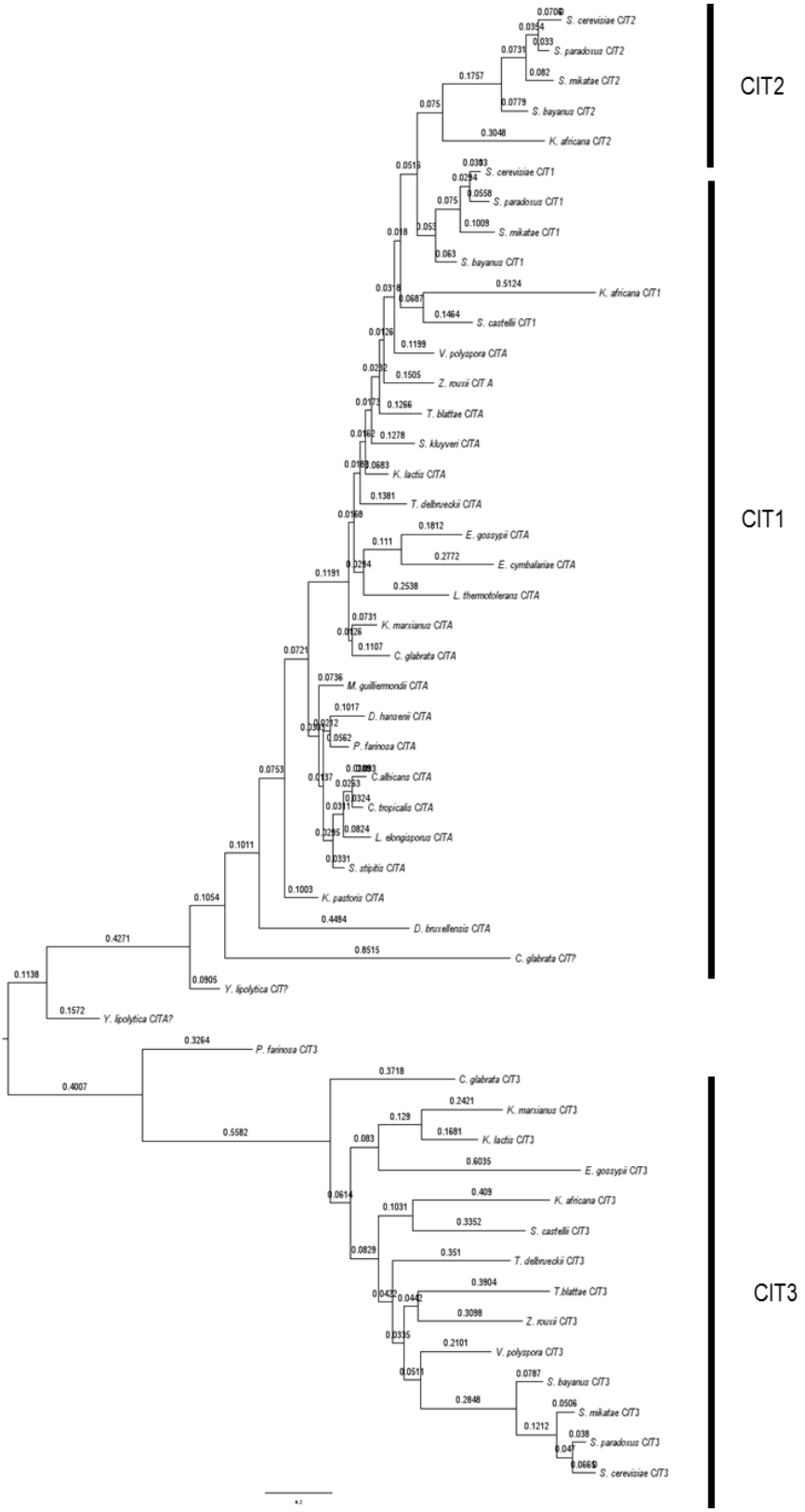


Figure 3.7: Phylogenetic tree of Citrate Synthase proteins in the Saccharomycotina *The branching of CIT1 and CIT2 from the ancestral 'CIT_A' is shown. Tree was constructed using maximum likelihood, and Jones-Taylor-Thornton substitution model. PhyML support values are shown for each branch.*

3.5 Discussion

The analyses presented in this chapter show that the Retrograde Response has a complex evolutionary history. Retrograde Signalling genes exhibit two broad patterns of phylogenetic distribution; those conserved across the Fungi and Metazoans, and those unique to the Ascomycota and Saccharomycotina. As shown in figure 3.5, possession of all three of the RTG genes is a trait unique to the Saccharomycotina. *RTG2* appears to have evolved following the divergence of the Taphrinomycotina from the rest of the Saccharomycotina and Pezizomycotina. In addition, *RTG2* appears to have been secondarily lost in the 'CTG clade' within the Saccharomycotina. *MKS1*, the primary negative regulator of the retrograde response is unique to the Saccharomycetaceaes; indeed it appears to be the sole member of a gene family that likely evolved de novo following the divergence of the Saccharomycetaceae from the CTG Clade.

The evolutionary history of genes regulated by the Retrograde Response was also investigated. The timing of the *PYC*, *DLD* and *CIT* gene duplication events appears consistent with the whole genome duplication, while the *ACO* and *IDH* most likely duplicated much earlier (Kellis, et al., 2004).

These results show two distinct regulators of Retrograde Signalling; a core group comprising the RTG genes and *MKS1*, found together exclusively within the Saccharomycetaceaes, and a highly conserved group of peripheral regulators that link Retrograde Signalling which are involved in multiple cellular processes.

Phylogenetic reconstruction of the Citrate Synthase genes in the Saccharomycotina suggests that *CIT1* and *CIT2* arose from an ancestral CIT gene (referred to as CIT_A). The timing of this duplication is consistent with the Saccharomycetaceae whole genome duplication (Kellis, et al., 2004). Under this interpretation *CIT2* has been lost from a small number of post-WGD species, including *V. polyspora*

and *S. castelli*. The third isoform of citrate synthase, *CIT3*, is absent from the CTG clade, apparently having been secondarily lost. *CIT3* is only distantly related to *CIT2* and *CIT1*. *S. cerevisiae CIT1* and *CIT2* have distinct functions and localizations; in the mitochondrial TCA cycle and the peroxisomal glyoxylate cycle, respectively. The duplication of each of these genes from the ancestral CIT_A gene appears to correlate with the emergence of a peroxisomal localization motif; SKL at the C-terminus of Cit2p. The evolution of *CIT2* and *CIT1* from CIT_A is further discussed in Chapter Four, section 4.3.3. The analyses presented in this chapter are limited by scant experimental work that has been done in other Saccharomycotina species. These results reflect gene distributions and phylogenetic results, rather than actual laboratory work. Thus, while the absence or presence of certain genes may be indicative that a particular function is not operating in a species, only genetic and biochemistry studies can conclusively determine the true extent of these processes.

Conclusions

The Retrograde Response has been posited as the basal form of several eukaryote stress response and longevity pathways (Srinivasan, et al., 2010)(Jazwinski & Kriete, 2012). The results presented in this chapter do not support this hypothesis. Rather, the Retrograde Response is comprised of two distinct groups of genes; the core RTG/*MKS1* regulators of the Retrograde Response, and the peripheral, highly conserved regulators. Likewise, genes expressed as a part of the Retrograde Response fall into two categories; those which undergo a shift in gene expression from HAP to RTG, and those exclusively regulated by the RTG genes. Genes in this latter group; *CIT2*, *PYC1* and *DLD2*, are paralogous to genes belonging to the former group. In effect, the Retrograde Response as it is understood in *S. cerevisiae*, is unique to the post-WGD Saccharomycetaceaes yeasts.

CHAPTER FOUR

Discussion

4.1 Introduction

As was laid out in Chapter One, the two aims of this thesis were to:

- To test if *TAR1* is a nuclear encoded modifier of mitotypic drive
- To determine how the yeast Retrograde Response evolved.

To test if *TAR1* was the hypothesized nuclear encoded response to selfish mitotypes, a series of experiments were conducted to test if deletion of *TAR1* affected the proportion of petite progeny following sexual reproduction between strains carrying ρ^- mitotypes, and strains carrying ρ^+ mitotypes. In order to accomplish the aim of determining how the Retrograde Response evolved, phyletic profiling of Retrograde Response signalling and target genes was carried out in order to show the conservation of these genes. In this final chapter the findings of this thesis are to be discussed in reference to one another, and to provide some context for how these results fit into our wider understanding of yeast evolution, cellular senescence and mitochondrial dynamics.

4.2 TAR1 depresses the drive of selfish mitotypes

As presented in Chapter 2, deletion of *TAR1* in the non-petite parent during sexual reproduction is correlated with an increased frequency of petite cells in the resulting diploid strain. (*See Chapter Two, Experiment Three*).This result is interpreted as a shift in petite frequency. Selfish genetic elements, such as ρ^- mitotypes, spread throughout a population by exhibiting drive. Drive describes a transmission rate during sexual reproduction of greater than 50%. A ρ^- mitotype that exhibited drive

of 100% would effectively result in uniparental inheritance of mtDNA, and thus 100% of progeny would be of the petite phenotype.

4.2.1 The Function of TAR1

The experimental conditions of both Experiment Two and Experiment Three more closely resemble the ecological conditions within which wild *S. cerevisiae* inhabits than the conditions of previous studies into ρ^- mitotypes and petites such as MacAlpine et al. (2001) and Blanc & Dujon, (1980). Wild *S. cerevisiae* inhabits rotting fruit. It is non-motile and may be transported between fruits by insects (Mortimer & Polsinelli, 1999). Once established on a fermentable carbon source, S. *cerevisiae* rapidly changes its environment through the production of ethanol. One effect of this "ecosystem engineering" is the elimination of competing microorganisms (Goddard, 2008). The petite phenotype shares several disadvantages with the microorganisms yeast ecosystem engineering evolved to eliminate, notably a reduced tolerance to high ethanol concentrations (Hutter & Oliver 1998). How petites respond to the other effects of *S. cerevisiae* ecosystem engineering such as increased osmotic pressure and temperature flux (which also enhances ethanol toxicity) remains untested. An 'engineered' environment, if populated by heteroplasmic cells, posits a strong selective advantage to a modifier to the drive of ρ^- mitotypes. Is *TAR1* such a modifier? The difference in petite frequency between crosses between petites and *TAR1* and *ΔTAR1* in Experiment Three supports this hypothesis.

The observed change in the drive of ρ^- mitotypes occurred despite *TAR1* being deleted in only the non-petite parent cell. That this resulted in a statistically significant increase in petite frequency may hint towards how *TAR1* modifies the drive of selfish mitotypes during sexual reproduction. It is

assumed that the petite parent carried a functional copy of *TAR1* within the rDNA array, which was expressed following activation of the yeast Retrograde Response. In addition, after karyogamy and the inheritance of ρ^- mitotypes, expression of *TAR1* would continue in the zygotic bud and its offspring. Deletion of *TAR1* from a single parent would leave only a narrow window during which the drive of the ρ^- mitotype would goes unmitigated between the movement of the mitochondrial network into the zygotic bud and the subsequent expression of *TAR1* from the petite inherited genome and its localization to the region of the mitochondrial inner membrane inherited from the *ΔTAR1* parent.

4.2.2 A plausible mechanism for TAR1

In *S. cerevisiae* mitochondria are actively moved along an actin filament from mother to daughter cell during budding reproduction (Vevea, et al., 2013). It is not known if this mechanism also operates to control the movement of mitochondria into the zygotic bud.

As a part of the mitochondrial nucleoid, Tar1p could depress the drive of ρ^- mitotypes by inhibiting the binding between the mitochondrial nucleoid and the actin cytoskeleton. This would prevent nucleoids carrying ρ^- mitotypes or nucleoids from petite cells, from being transported into the zygotic bud. However, this mechanism is inconsistent with results showing that deletion of *TAR1* does not increase the frequency of petites in vegetative growing haploid cells as shown in Experiment One. If Tar1p did inhibit the movement of nucleoids carrying Rho- mitotypes, then it would be expected that the deletion of *TAR1* would result in an increased frequency of petites as selfish mitotypes would no longer be preferentially retained by the mother cell. Such a mechanism for Tar1p would also be

inconsistent with results presented in Experiment Three in Chapter 2 showing that the deletion of *TAR1* in the respiration competent parent still results in an increased drive of selfish mitotypes. Likewise, if Tar1p served to inhibit the replication of hypersuppressive mitotypes within the cell, it would be anticipated that the frequency of petites arising in haploid *ΔTAR1* cells would be higher, though this is not observed in Experiment One.

One mechanism through which Tar1p could suppress the drive of ρ^- mitotypes during sexual reproduction while consistent with the results from Experiment Three, while not affecting the formation and drive of respiration incompetent mitotypes during vegetative growth could be through inhibition of mtDNA recombination. The mitochondrial DNA in *S. cerevisiae* is highly recombinogenic. Following cell fusion and the mixing of the mitochondria network during sexual reproduction, mtDNA molecules are nearly completely hybridized within two generations (Shannon, et al., 1972). Recombination between ρ^- and ρ^+ mitotypes may result in the loss of respiratory competence within the cell. If Tar1p slowed the rate of mtDNA recombination; either through interacting with the mtDNA molecule itself, the mitochondrial nucleoid, or some other component of the mitochondrial inner membrane, then there would be a higher probability of ρ^+ homoplasmic daughter cells being produced in early generations of the diploid strain.

An anti-recombination role for Tar1p would also fit with its expression as a part of the Retrograde Response in ageing cells. The Retrograde Response is activated as the cell ages in response to accumulative oxidative damage to mitochondrial components resulting in a decline of mitochondrial membrane potential (Jazwinski, 2005). Fusion between different regions of the mitochondrial network occurs in respiring cells to maintain homeostatic health (Devin & Rigoulet, 2004). Fusion events would also provide for the mixing of different mitotypes within the cell. In such conditions, mixing of

lipids and proteins would be beneficial, while the mixing of mtDNA molecules may be deleterious. *TAR1* expression has been identified during respiration (Bonawitz et al., 2008), and respiratory activity is correlated with increased interconnectivity of the mitochondrial network. An anti mtDNA recombination agent would mitigate the spread of damaged mitotypes through recombination in respiring cells as well.

In *S. cerevisiae* cells utilizing fermentable carbon sources, reactive oxygen species are produced at a greatly reduced rate (Landolfo, et al., 2008). In addition, the mitochondrial network in non-fermenting cells remains relatively fragmented (Rafelski, 2013), thus limiting the potential for mtDNA recombination. If *TAR1* does serve to act as an anti-recombination protein, then deletion of *TAR1* would not result in an increased petite frequency in cells growing on fermentable carbon sources, as those were in Experiment One.

Inhibition of mtDNA recombination in cells replete with damaged mitochondria would also serve a positive role in petite-negative *K. lactis*, which possess a bona fide *TAR1* orthologue (Galopier, et al., 2011).

A number of experiments could be conducted to test if Tar1p inhibits mtDNA recombination. One way to test this mechanism would be to identify the rate of mixing of markers from each mitotype following crosses in each of a *ΔTAR1* and *TAR1* background.

4.3 The Retrograde Response is unique to the Saccharomycetaceae yeasts

4.3.1 Phyletic Profiling indicated the Retrograde Signalling pathway is recently evolved

In Chapter 3 phyletic profiling was carried out to determine the evolutionary history of the genes involved in the Retrograde Response. In Figure 3.5 of Chapter Three, these results are presented. The phylogenetic distribution of genes involved in the *S. cerevisiae* Retrograde Signalling pathway suggests that this pathway has evolved recently.

The core Retrograde Signalling pathway genes; *RTG1*, *RTG2* and *RTG3* appear to be unique to the Ascomycota. An inhibitor of the Retrograde Response, *MKS1*, is even more restricted, apparently unique Saccharomycetaceae yeasts. Conversely, genes associated with stress response and nutrient sensing pathways, including *TOR1*, *RAS2* and *BMH1* are conserved with metazoan homologues identified. Each of these genes have undergone a duplication event though the diversity of functions each carries out makes it impossible to determine if this duplication is relevant to their function within the Retrograde Signalling pathway. Other genes, including *GRR1* and *LST8* lack duplicates, but do have mammalian homologues.

4.3.2 Evolution of the Petite Phenotype & Retrograde Response

Fekete, et al., (2007) carried out an extensive investigation into the distribution of the petite phenotype among the Saccharomycetaceae species. Their data show that post-whole genome duplication species are nearly all petite positive, while pre-whole genome duplication species are primarily petite negative, with a 'mosiac' distribution of petiteness observed in those species immediately before, or after the duplication event occurred. It is worth noting that the post-whole genome duplication Saccharomycetaceae *V. polyspora* was selected for phyletic profiling in **section X**, however it is an anomaly in terms of being one of only two petite negative post-WGD Saccharomycetaceae yeasts out of 40 discovered in the Fekete, et al., (2007) survey. The distribution of the petite phenotype outside of model organisms and the Saccharomycetaceaes remains poorly understood. This is further complicated by still cited early research which conflated respiration incompetence in fungi species with the ρ^- and ρ^0 induced petite phenotype of *S. cerevisiae* (Brewer & Rusch, 1965) (Hottinguer-de Margerie & Moustacchi , 1975). On the other hand, respiration incompetent mammalian cells replete with "ρ^0" mitotypes have been observed (King & Attardi 1989). Outside of the Saccharomycotina the petite phenotype appears to occur extremely infrequently or not at all without artificial inducement (Haffter & Fox 1992). In contrast, the petite phenotype arises at a frequency of 5-10% in *S. cerevisiae* growing on fermentable carbon sources (Chen, X. J., & Clark-Walker , 1999), (Experiment One, Chapter Two). Thus it seems most useful to understand petiteness as a uniquely Saccharomycotina phenomenon, in which the mtDNA is spontaneously lost or extensively deleted resulting in respiration incompetence.

4.3.3 The Function of the Citrate Synthase isoforms

CIT2 is upregulated as a part of the Retrograde Response. Three isoforms of citrate synthase are found in *S. cerevisiae*. Phylogenetic analyses determined that *CIT1* and *CIT2* are paralogs, and descended from a common ancestor, referred to as CIT_A. *CIT3* is more distantly related as shown in Figure 3.7. in Chapter 3. In *S. cerevisiae CIT2* possess a C-terminus peroxisomal localization sequence (SKL). This motif is used to detect and transport *CIT2* into the peroxisome, where it catalyzes the condensation of acetyl-CoA and oxaloacetate into citrate as a part of the glyoxylate cycle. *CIT1* and *CIT3* are localized to the mitochondria, where *CIT1* catalyses the same reaction as a part of the TCA cycle.

The timing of the Saccharomycetaceae whole genome duplication event aligns with the timing of the duplication of CIT_A into *CIT1* and *CIT2* (Souciet, et al., 2009). This whole genome duplication event resulted in the duplication of all genes in the ancestor. However, not all duplicates were retained. Selective pressures may have operated to ensure that both *CIT1* and *CIT2* were retained. Previous research has identified a cryptic peroxisomal localization motif towards the N-terminus in *CIT2* in addition to the SLK peroxisomal localization motif found at the C-terminus (Lee, et al., 2000). Multiple sequence alignment of Citrate Synthase genes in the Saccharomycetes yeasts shows that this cryptic signal is also found in pre-whole genome duplication taxa (Lee, et al., 2000). Duplication of these genes therefore, may have allowed for the subfunctionalization of these processes.

Both *CIT1* and *CIT2* are subject to Retrograde Response regulation. However, only *CIT1* is subject to regulation under normal respiratory metabolism (Traven, et al., 2001). *CIT2* is not transcriptionally activated during such conditions (Traven, et al., 2001), and is exclusively expressed during the Retrograde Response (Epstein, et al., 2001). The presence of the core Retrograde Signalling pathway

genes predates the duplication of CITA into CIT1 and CIT2 (*see figure 3.5, Chapter 3*) In addition, the *E. gossypii* orthologue of *RTG2* is able to activate the Retrograde Response when expressed in *S. cerevisiae (*Ünlü, et al., 2013). Thus there is strong evidence that the Retrograde Response exists in the pre-whole genome duplication yeasts and that CIT_A would be subject to the Retrograde Response.

The duplication of *CIT1* and *CIT2* may have allowed for the evolution of differentially expressed paralogs; *CIT1* constitutively regulated by the Hap2-5 complex during respiration, and by Rtg1p/Rtg3p during respiration incompetence and *CIT2* expressed exclusively during the Retrograde Response under conditions of respiration incompetence. This pattern is seen with the non-duplicated *ACO1*; which is controlled by the Retrograde Response during respiration dysfunction, and the Hap2-5 complex during respiration competence (Epstein et al., 2001).

Previous research has shown the important role that gene duplication has had on *S. cerevisiae* metabolic pathways (Papp, et al., 2004). One example is the Duplication of *ADH1* and *ADH2*, which has had the effect of increasing the efficiency of the 'make-accumulate-consume' strategy which *S. cerevisiae* employs (Piskur, et al., 2006). Each paralog of ADH has a different enzymatic efficiency for each direction of the reaction, implying that they have undergone, or are undergoing a process of neofunctionalization. This change in efficiency may occur because the necessary changes to the enzyme structure to increase the efficiency of both reactions (forward and reverse) may be mutually exclusive (Piskur, et al., 2006). Both CIT1 and CIT2 catalyze the same reaction. However both are localized to different parts of the cell. Duplication may have paved the way for the adoption of the SKL peroxisomal localization motif in CIT2, without interfering with CIT1 localization. Similarly differential expression ensures that the glyoxylate cycle isoform of citrate synthase isn't expressed under conditions in which it would not be advantageous to, such as during respiratory growth.

4.4 *TAR1* and the Retrograde Response are linked

TAR1 and the Retrograde Response are both adaptations to mitochondrial dysfunction. Results presented in Chapter Two show that *TAR1* functions to ameliorate the drive of ρ^- mitotypes during sexual reproduction. *TAR1* expression is induced through the activation of the Retrograde Response, which lifts Sir2p suppression of Pol II silencing at the rDNA locus and triggers the formation of ERCs. The Retrograde Response also involves the expression of genes involved in anaplerotic pathways, which compensate for the loss of function of succinate dehydrogenase, a consequence of loss of mtDNA encoded genes. The Retrograde Response thus alleviates the loss of mitochondrial function associated with selfish mitotypes, as well as suppressing their ability to spread into the next generation during sexual reproduction.

4.5 Conclusions

As stated in the beginning of Chapter One, the aims of this thesis were to:

- To test if TAR1 is a nuclear encoded modifier of mitochondrial inheritance
- To determine how the yeast Retrograde Response evolved.

To accomplish these aims the effect of *TAR1* deletion on spontaneous petite formation, and the drive of ρ^- mitotypes was measured. These results showed that deletion of *TAR1* was correlated with an increased diploid petite frequency, suggesting that *TAR1* modified the drive of selfish mitochondrial haplotypes. A function for TAR1 in inhibiting the recombination of mtDNA following sexual reproduction was hypothesized; which would be consistent with the results presented in Chapter Two.

In Chapter Three, the evolution of the Retrograde Response was investigated. Phyletic Profiling showed that the central regulators of the Retrograde Signalling pathway; RTG1, RTG2, RTG3 and MKS1, are only found together in the Saccharomycetaceae yeasts. The prototypical Retrograde Response gene, *CIT2*, was found to have undergone duplication and retention as a part of the whole genome duplication that took place within the Saccharomycetaceaes. This suggests that the evolution of the Retrograde Response is a novel regulatory pathway in the Saccharomycetaceae yeasts, and has possibly undergone a significant change in function following the recent whole genome duplication.

Ultimately, these results show that the *S. cerevisiae* Retrograde Response has two key roles in relation to selfish mitochondrial DNA. Firstly, it compensates for the loss of metabolic function that respiration incompetent mitotypes induce. Secondly, it regulates the expression of *TAR1*, a gene that suppresses the drive of selfish mitochondria.

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